Studies of the PI3K/Akt and MAPK/ERK1/2 signaling pathways: identification of novel downstream targets involved in development and progression of melanoma

by

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1. AIMS OF THE STUDY

Malignant melanoma is an aggressive cancer form with few treatment options and poor survival for patients with advanced disease. Increasing knowledge about molecular changes leading to development and progression of melanoma has identified the PI3K/Akt and MAPK/ERK1/2 pathways as frequently deregulated mediators of the malignant phenotype. Although considerable effort is made to therapeutically target these pathways, desired clinical results are yet to be obtained. For this reason there is still a need for more in depth dissection of these pathways and identification of novel downstream targets, which could hopefully lead to improvement of diagnostic methods and design of new and more efficient therapeutic strategies. With this in mind the aims of the present study were to:

1. Investigate activation status of the PI3K/Akt pathway in melanoma specimens and verify its significance in relation to clinicopathological parameters and patient outcome.

2. Identify and characterize new downstream targets of the PI3K/Akt and MAPK/ERK1/2 pathways involved in development and progression of melanoma.

2. LIST OF PUBLICATIONS

I **Slipicevic A,** Holm R, Nguyen MT, Bøhler PJ, Davidson B, Flørenes VA: Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome. Am J Clin Pathol. 2005 Oct;124(4):528-36.

II **Slipicevic A,** Jørgensen K, Skrede M, Ree Rosnes AK, Trøen G, Davidson B and Flørenes VA: The fatty acid binding protein 7 (FABP7) is regulated independently by PKC and the MAPK/ERK pathway and is involved in proliferation and invasion of melanoma cells. BMC Cancer. 2008 Sep 30;8:276

III Øy GF, **Slipicevic A,** Davidson B, Solberg Faye R, Mælandsmo MG, Flørenes VA. Biological effects induced by insulin-like growth factor binding protein 3 (IGFBP-3) in malignant melanoma. Int J Cancer. 2009 Jul 8;126(2):350-361

3. INTRODUCTION

3.1. Cancer

Cancer is thought to arise through clonal expansion of a single cell that has initially acquired heritable changes in its DNA. Further accumulation of genetic alterations provides progeny cells with selective growth advantages leading to an increase in cell number ultimately giving rise to a tumor (1;2). Genetic abnormalities found in cancer include point mutations, gene deletions, amplifications, translocations, inversions or duplications and loss of whole chromosomes. Additionally, epigenetic mechanisms like hypermethylation, acetylation and genomic imprinting also play a central role in development and progression of cancer. Common for all these genetic alterations is that they affect two general classes of genes: proto-oncogenes and tumor suppressor genes. Activated oncogenes promote proliferation and survival while tumor suppressor genes negatively regulate these processes. DNA repair or stability genes are a third class of genes altered in cancer. This group is essential for maintenance of genome integrity by keeping genetic alterations in the cell to a minimum (3;4). In addition to loss of growth control and resistance to apoptosis (programmed cell death), cancer cells acquire a variety of special characteristics which define the malignant phenotype (hallmarks of cancer), including an extended or indefinite replicative potential (replicative immortality), genomic instability, ability to attract or create blood supply (angiogenesis), ability to invade the surrounding tissue and ability to survive and proliferate in an ectopic environment forming metastasis (Figure 1)(5).

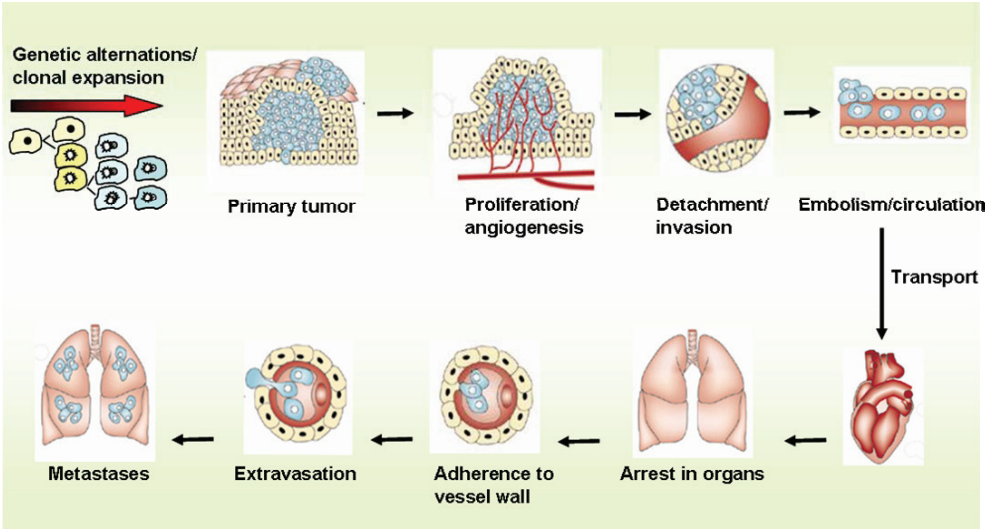

Figure 1. Cellular transformation and the main steps in the metastatic process. Clonal expansion of genetically altered cell generates new subclones, which accumulate additional genetic changes providing them with growth advantage, ultimately leading to formation of a primary tumor mass. Further acquisition of malignant features allows tumor cells to fulfill the metastatic process. Adapted by permission from Macmillan Publishers Ltd : ref. (6), © 2003

3.2. Cell cycle

The basic function of the cell cycle is to accurately duplicate DNA and segregate the copies into two genetically identical daughter cells. The cell cycle consists of four phases: the S phase when DNA replication (synthesis) occurs, the M (mitosis) phase when identical chromosome copies are distributed to two new daughter cells and the two gap phases, G1 and G2, in which the cells are allowed to grow and prepare for the upcoming events of S and M, respectively (7). Progression through the G1 phase is highly dependent on extracellular signals, stimulating the cells to overcome the so called “restriction point” in late G1 (8). After the restriction point, the cells become independent of external mitogenic stimuli and can complete the cell cycle autonomously.

Transition through the cell cycle is driven by activated cyclin-dependent kinases (CDKs) and their activating cyclin subunits (Figure 2). CDK/cyclin complexes phosphorylate and inactivate the retinoblastoma protein (pRb) (9). When pRb is in a hypophosphorylated (active) state, it binds E2F transcription factors, thereby preventing expression of genes essential for progression from G1 into the S phase (10-12). Phosphorylation (inactivation) of

pRb liberates E2F, thus allowing cell cycle progression to occur. CDK activity is regulated by two families of inhibitors, INK4 and Cip/Kip (13). INK4 proteins, including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p14^{INK4D/ARF}$ specifically bind and inhibit CDK4 and CDK6. $p14^{INK4D/ARF}$ also binds directly to MDM2, resulting in stabilization of the tumor suppressor protein p53 (14). p53 has an important function in maintaining the integrity of the genome by inducing cell-cycle arrest or programmed cell death following DNA damage. The Cip/Kip family, composed of $p21^{Cip1/WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$ proteins, binds to and inhibit the activation of CDK/cyclin complexes (15).

Most human cancers have abrogations in genes that directly or indirectly regulate the cell cycle. The most frequently occurring are mutations in *RB* and *TP53* genes as well as inactivation of $p16^{INK4A}$, $p15^{INK4B}$ and $p27^{Kip1}$ proteins (16;17).

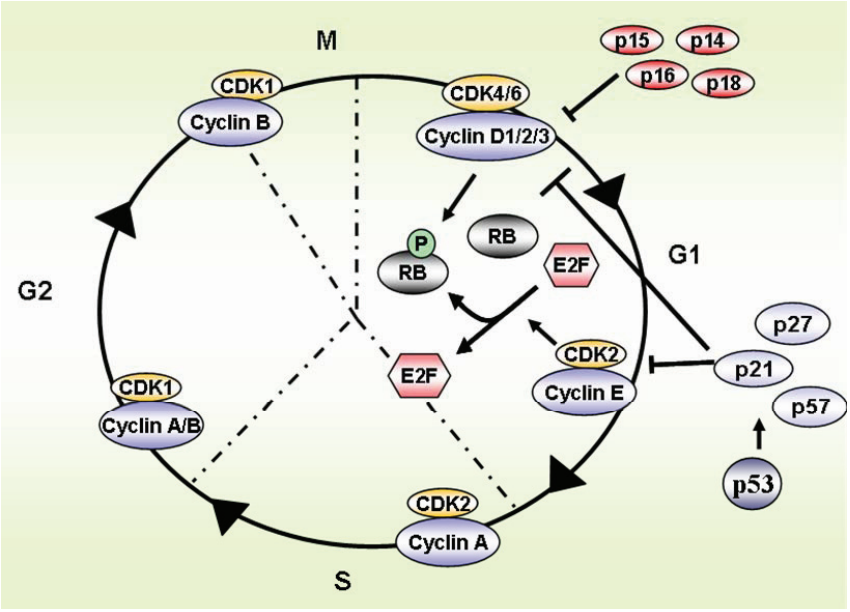

Figure 2. Regulation of the mammalian cell cycle, with emphasis on G1-S transition.

3.3. Apoptosis

Cells can, upon receiving proper signals, activate intracellular death programs which in a sequence of controlled steps leads to locally and temporally defined self-destruction (18;19). Programmed cell death has a large biological importance, contributing to homeostasis, development, differentiation, regulation and function of the immune system and elimination of abnormal, misplaced and nonfunctional cells (20). One form of programmed cell death is termed apoptosis, characterized morphologically by cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and disassembly into membrane-enclosed vesicles followed by engulfment by resident phagocytes (*in vivo*) (19;21). Apoptosis is triggered by various stimuli from outside or inside the cell, e.g. by developmental death signals or irreparable DNA damage caused by treatment with cytotoxic drugs or irradiation.

The molecular execution of apoptosis involves activation of members of a family of cystein-dependent aspartate-specific proteases (caspases) that cleaves substrates after aspartic acid (asp) residues (22). Two major apoptotic pathways lead to caspase-activation (Figure 3). The extrinsic pathway is initiated through stimulation of transmembrane death receptors by ligands such as Fas, TNF-α and TRAIL (23). Ligand binding leads to receptor clustering and association of the adaptor protein Fas-associated death domain (FADD) and the initiator caspases 8 or 10, forming a death-inducing signaling complex (DISC) (24-26). This complex brings procaspase molecules in the proximity of one another, facilitating their autocatalytic processing and release into the cytoplasm. Here they activate effector caspases 3, 6, and/or 7, responsible for substrate cleavage and appearance of apoptosis–related morphological changes (27). Formation of DISC and activation of caspases can be modulated by c-FLICE inhibitory protein (c-FLIP), which can interact with FADD to block initiator caspase activation. Additional inhibitory mechanism includes decoy receptors, which can block ligand binding or directly abrogate pro-apoptotic receptor stimulation (28).

The intrinsic pathway, also referred to as the mitochondrial pathway, is initiated by release of cytochrome *c* from the mitochondria to the cytoplasm. Released cytochrome *c*, in turn, forms a complex with Apaf-1 and caspase 9, which subsequently activates caspase 3. The release of cytochrome *c* is regulated by Bcl-2 proteins, a family of proteins including both pro-apoptotic (Bax, Bim, Bad, Bak, Bid, Bcl-$_X$S, Noxa, Puma) and anti-apoptotic members

(Bcl-2, Bcl-$_X$L, Mcl-1) (29). The Bcl-2 proteins are regulated mainly through dimerization, translocation and phosphorylation (30).

Considerable crosstalk exists between the extrinsic and intrinsic pathways. Thus, caspase 8 can cleave Bid to tBid, which acts as a signal on the membrane of mitochondria to facilitate release of cytochrome *c* (31). Deregulation of the apoptotic process contributes to tumor initiation as well as progression and treatment resistance (32;33). Tumors often become resistant to apoptosis by overexpressing members of the Bcl-2 family of pro-survival proteins. In addition, loss of p53 regulated pro-apoptotic genes (e.g., *Bax, Noxa, Puma, Bid, CD95, APAF-1, DR5, p53AIP*) are often observed (34;35) .

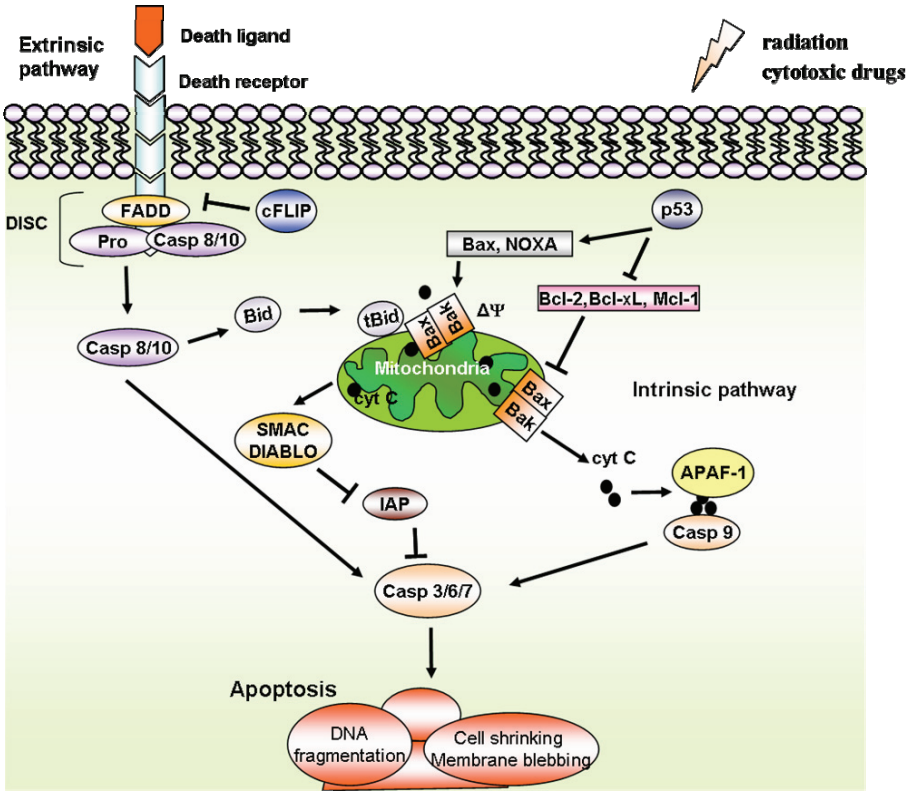

Figure 3. The extrinsic and intrinsic apoptosis signaling pathways.

3.3.1. Anoikis

In most non-transformed cell types, adhesion to extracellular matrix (ECM) is required for cellular survival, and disruption of such interaction leads to a specific type of apoptosis known as anoikis (Greek for homelessness) (36) *In vivo*, anoikis prevents detached cells from reattaching to new matrices and growing dysplastically. During normal skin renewal, keratinocytes undergo anoikis as they loose anchorage to the substratum and eventually are shed from the skin (37). In contrast to normal cells, most tumor cells become anoikis resistant, making the cells able to leave the primary tumor and subsequently metastasize (38).

Anchorage of cells to components of the ECM like fibronectin, collagens, and laminin is mainly mediated by integrins, which are transmembrane cell surface receptors. Integrins mediate their signals via integrin-associated non-receptor kinases of which focal adhesion-kinase (FAK) and integrin-linked kinase (ILK) are the best characterized (39). Upon integrin ligation, the integrin/FAK signaling complex activates several survival signaling pathways inside the cells, including the phosphoinositide-3 kinase (PI3K)/Akt and the p42/p44 mitogen-activated protein kinase/extracellular signal-regulated kinases 1/2 (MAPK/ERK1/2) pathways (40;41).

Anoikis is essentially an apoptotic process and its execution involves both caspases and proteins of the Bcl-2 family (42;43). Loss of ECM contact leads to increased accumulation of pro-apoptotic Bim due to inhibition of the PI3K/Akt and MAPK/ERK1/2 pathways which under normal conditions phosphorylate Bim marking it for proteasome-dependent degradation (44). Bim is usually sequestered in the dynein complex, but upon loss of integrin engagement it translocates to the mitochondria and interacts with Bcl-$_X$L, neutralizing its pro-survival function (45). Thus, constitutively active survival pathways or changed pattern of integrin expression makes cancers usually resistant to anoikis.

3.4. Cell signaling

Normal cellular homeostasis is dependent on the ability of cells to perceive and correctly respond to their microenvironment. Extracellular and intracellular signals are converted to an adequate cellular response like cell division, cell cycle arrest or apoptosis through complex networks of signaling cascades. Many extracellular signals are transmitted to the cells via cell surface receptors. Ligand binding to receptors usually leads to conformational changes and phosphorylation of downstream effectors.

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3.4.1. The PI3K/Akt pathway

The PI3K/Akt pathway regulates a number of cellular processes, including metabolism, growth, proliferation, apoptosis and cell migration (Figure 4) (46).
PI3Ks are a family of intracellular lipid kinases divided into three major classes (I, II and III) according to their structure and substrate specificity. Class I PI3Ks are coupled to external stimuli and transmit signals from receptor tyrosine kinases (RTK), integrins and G-protein coupled receptors. In addition, PI3Ks are also activated by intracellular proteins such as PKC, Rac, Rho and Src (47). PI3K catalyzes phosphorylation of inositol-containing lipids, known as phosphatidylinositols (PtdIns). Its primary *in vivo* substrate is phosphatidylinositol-4, 5-bisphosphate (PIP2) which is converted to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), an important second messenger molecule. PIP3 serves as a docking site for subsets of proteins containing pleckstrin homology domains which are recruited to the plasma membrane and activated.

Among major downstream targets of PI3K is the serine/threonine kinase Akt (PKB). Translocation of Akt to the membrane brings it close to upstream regulatory kinases such as the phosphoinositide dependent kinase 1 (PDK1) that phosphorylates Akt on Thr 308, which is necessary for Akt activation. However, maximal activation requires additional phosphorylation at Ser473 by the rapamycin-insensitive mTOR complex (mTORC2) (48).
Activated Akt has been demonstrated to phosphorylate pro-apoptotic Bad leading to its degradation as well as caspase 9, which inhibits its catalytic activity, thereby protecting the cells from apoptosis. Furthermore, Akt can influence cell survival by indirectly effecting nuclear factor of κB (NF-κB), a central regulator of cell death. Activation of Akt promotes multiple effects on cell cycle regulation through phosphorylation and inactivation of the cell

cycle regulators, $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ and by preventing degradation of cyclin D1 through inactivation of glycogen synthase kinase 3β (GSK-3β) (49-51).

The PI3K/Akt pathway is negatively regulated by the tumor suppressor protein PTEN (Phosphatase and tensin homologue deleted on chromosome 10) (52). PTEN is as a dual specificity lipid and protein phosphatase which dephosphorylates PIP3. Cells lacking PTEN have elevated levels of PIP3 and phosphorylated Akt, making the PI3K/Akt pathway constitutively active. PTEN has also been demonstrated to associate with p53 and increase its protein levels and activity (53). Furthermore, PTEN is suggested to restrain cell migration, an effect dependent on its protein phosphatase activity (54). Most recently, it has been shown that PTEN can regulate the c-Jun-N-terminal Kinase (JNK)/stress-activated protein kinase (SAPK) pathway in an Akt-independent manner (55).

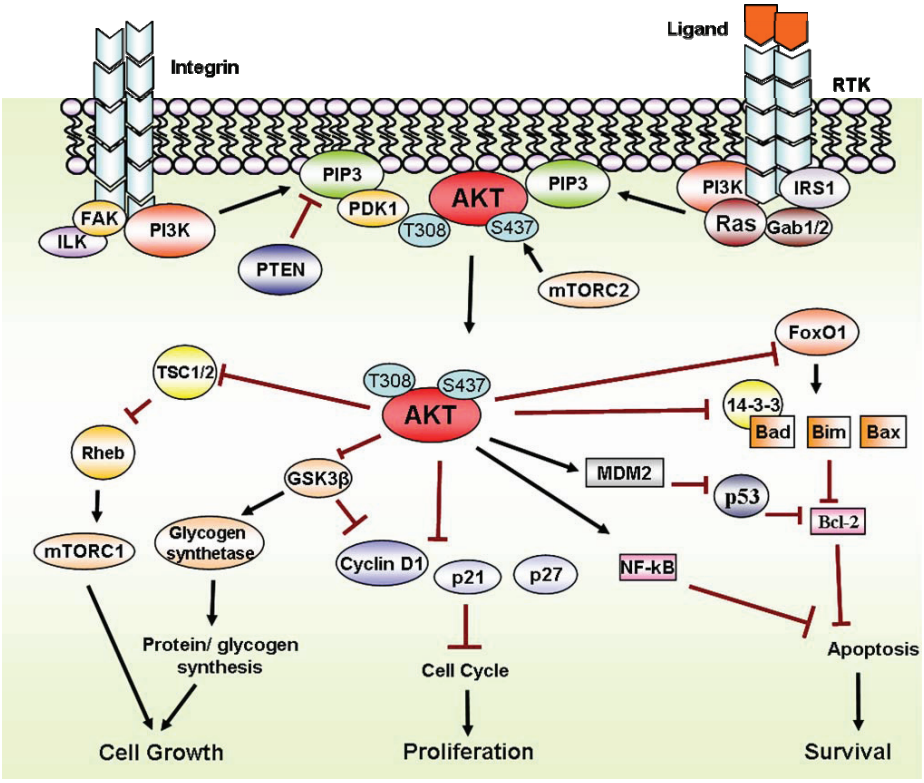

Figure 4. The PI3K/Akt pathway.

3.4.2. MAPK pathways

Activated cell surface receptors can transmit signals through engagement and activation of the MAPK family of protein serine/threonine kinases. The major MAPK pathways include the MAPK/ERK1/2, JNK/SAPK and p38-MAPK pathways (Figure 5). Each of these pathways is a signaling cascade consisting of a MAPK kinase kinase (MAPKKK or MEKK), a MAPK kinase (MAPKK or MEK) and a MAP kinase (MAPK), all activated by phosphorylation.

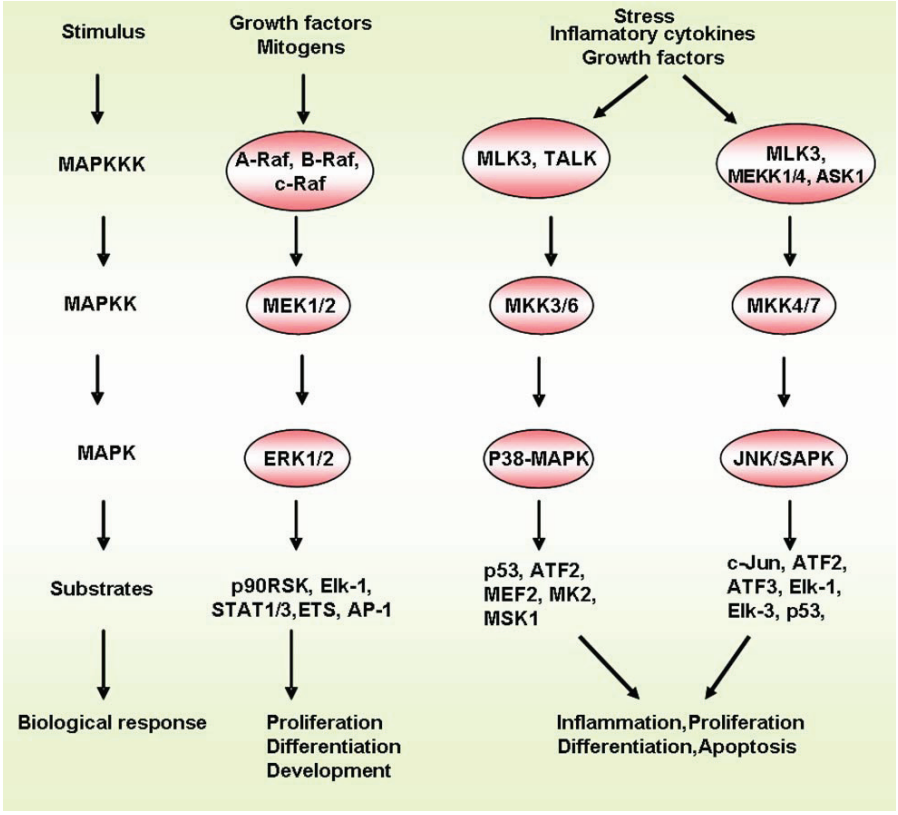

Figure 5. Major MAPK signaling cascades in mammalian cells.

3.4.2.1. The MAPK/ERK1/2 pathway

The MAPK/ERK1/2 cascade is comprised of Raf, MEK1/2 and ERK1/2 kinases (Figure 6). Activation of RTK leads to binding of adaptor molecules containing SH2 domains like Grb2 and Shc linking the receptors to a proline-rich region of the guanine nucleotide exchange protein SOS (son of sevenless), which stimulates the exchange of GDP for GTP on the Ras protein (56). In its GTP-bound state, Ras binds to Raf, bringing it to the plasma membrane where its protein kinase activity is increased and downstream kinase cascade is activated (57-59).

There are three known mammalian Raf isoforms; A-Raf, B-Raf and C-Raf/(Raf-1), translated from distinct genes on different chromosomes with tissue-specific expression and subcellular localization (60). Mutations increasing the catalytic activity of the Raf proteins have been identified in a number of human tumors. However, mutations in B-Raf occur most frequently (61). Raf phosphorylates and activates serine/threonine protein kinases MEK1 and MEK2 who subsequently phosphorylate ERK1 and ERK2 on tyrosine/threonine residues (62-64). In addition, Raf has been shown to interact with other targets including cell cycle regulators (e.g., pRb, Cdc25), apoptosis modulators (e.g., Bcl-2 proteins), apoptosis signal-regulating kinase 1 (ASK1) and translation regulators (e.g., eEF-1A), suggesting that Raf can modulate cellular processes through MEK1/2/ERK1/2 independent mechanisms (65-69).

Activated ERK1/2 phosphorylates numerous substrates in all cellular compartments thereby regulating proliferation, differentiation and cell survival. By activating the transcription factors AP-1 and ETS, ERK1/2 has been shown to regulate cyclin D1 transcription (70;71). Furthermore, physical interaction of CDK2 with ERK1/2 leads to nuclear translocation of CDK2, facilitating G1/S transition (72). ERK1/2 has also been shown to phosphorylate and modulate $p27^{Kip1}$, promoting its degradation, which again results in release of active CDK2/cyclin E complex and entry into the S-phase (73).

Cellular response to ERK1/2 activation is determined by duration, magnitude and subcellular localization of activated ERK1/2 (74). In some cell types sustained activation of ERK1/2 is required to induce proliferation while others require only transient activation (75-77). Furthermore, while moderate levels of ERK1/2 activity have been shown to induce

expression of cyclin D1 and cyclin E leading to accumulation of active CDK complexes, high levels of ERK1/2 activity influence the CDK inhibitor $p21^{Cip1/WAF1}$ by transcriptional as well as post-translational mechanisms, reducing CDK activity and inducing G1 arrest (78).

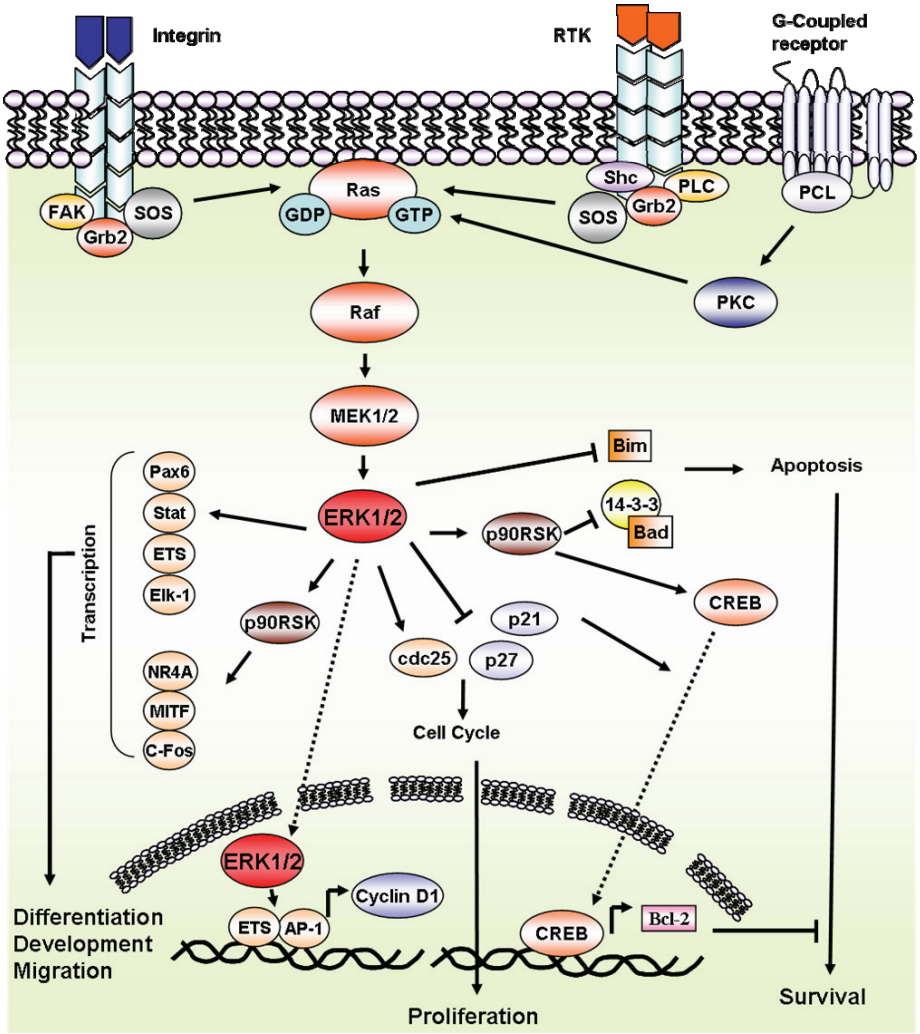

Figure 6. The MAPK/ERK1/2 pathway.

The MAPK/ERK1/2 pathway may either enhance or decrease sensitivity to apoptosis depending on the stimuli and cell type (79;80). Phosphorylation of Bad by ERK1/2

facilitates its binding to 14-3-3-protein, thereby suppressing its pro-apoptotic activity. Phosphorylation of Bim, on the other hand, promotes its degradation opposing apoptosis. Furthermore, ERK1/2 can increase expression of several pro-survival Bcl-2 proteins, including Bcl-2, Bcl-$_X$L and Mcl-1 (81). By inducing *MDM2* transcription and thereby p53 degradation, ERK1/2 can also regulate p53 mediated apoptosis (82).

MAPK/ERK1/2 signaling depends on phosphorylation on both tyrosine and threonine residues on ERK1/2, and removing phosphate from either site will ultimately lead to their inactivation. Such dephosphorylation is performed by a family of dual specificity phosphatases known as MAPK phosphatases (MKPs) (83). Serum and growth factors induce expression of certain MKPs, which in some cases contain ERK1/2-sensitive promotor elements, indicating that induction of MKPs may create a negative feedback mechanism, limiting the time course of ERK1/2 activation.

3.4.2.2. The JNK / SAPK and p38-MAPK pathways

When exposed to stress, including UV irradiation, osmotic stress, protein synthesis inhibitors or inflammatory cytokines, cells can activate the stress-activated protein kinases JNK/SAPK and/or p38-MAPK (Figure 5). As ERK1/2, also JNK/SAPK and p38-MAPK are activated by MAPKKs through phosphorylation on tyrosine and threonine residues. While MKK4 and MKK7 phosphorylate JNK/SAPK, p38-MAPK is phosphorylated by MKK3 and MKK6 (84).

A major downstream JNK/SAPK target is the transcription factor activator protein-1 (AP1), which is composed of Fos and Jun family members. Phosphorylation of the c-jun transcription factor by JNK/SAPK, leads to increased expression of numerous genes with AP1 sites in their promoters (85). Among other JNK/SAPK targets are the transcription factors ATF2, Elk1, c-myc and p53, all positive regulators of the transcription factor c-fos, further increasing the AP1 level (86).
Through phosphorylation, activated p38-MAPK regulates p53 and activating transcription factor 2 (ATF2), as well as protein kinases, including MAPK-activated kinase 2 (MK2) and mitogen-and stress-activated protein kinase 1(MSK1) (87).

Previously it has been anticipated that the MAPK/ERK1/2 signaling pathway mediates cell proliferation and survival because of its response to mitotic signals and proliferative cytokines, whereas activated JNK/SAPK and p38-MAPK have been suggested to play a role in apoptosis. However, also JNK/SAPK and p38-MAPK can regulate diverse cellular responses including cell cycle progression, survival and differentiation, depending on the stimuli and the strength and duration of their activation. In addition, recent studies have shown that MAPKKKs, believed to specifically activate the JNK/SAPK and p38-MAPK signaling pathways, also can activate MEK1/2, suggesting that different MAPK pathways affect each other through cross-talk reactions and feedback mechanisms (88).

3.5. IGFBP-3

Among receptors known to activate the PI3K/Akt and MAPK/ERK1/2 pathways is insulin-like growth factor 1 receptor (IGF-1R). It is activated by IGF-1 and by the related growth factor IGF-2, leading to phosphorylation of downstream adaptor proteins belonging to the insulin receptor substrate (IRS) family or src homologous and collagen (SHC) proteins (89). The activity of IGF-1R is regulated by the availability of its ligands. In serum and extracellular fluids, IGFs associate with IGF binding proteins (IGFBPs) which comprise a family of six related secreted proteins that specifically bind IGFs with high affinity. Of these, IGFBP-3 has the highest affinity for IGF-1 and is also the most abundant IGFBP family member in the circulation. Binding of IGF-1 to IGFBP-3 sequesters IGF-1 leading to prevention of IGF-1-induced IGF-1R autophosphorylation and signaling (90). In contrast, binding of IGFBP-3 to extracellular matrix or cell surfaces through the glycosaminoglycan-binding domain, decreases the affinity of IGFBP-3 for IGF-1, thereby increasing the level of free IGF-1 and receptor activation. IGFBP-3 may also enhance IGF-1 activity by slowly releasing IGF-1 to its receptor and inhibit IGF-R down-regulation normally caused by sustained IGF-1 exposure (91).

Expression of IGFBP-3 is regulated by interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-ß) and retinoic acid, as well as by IGF-1 (90). Also hypoxia can induce IGFBP-3 mRNA through p53-independent and dependent mechanisms. Moreover, signaling through the PI3K/Akt and MAPK/ERK1/2 pathways has been shown to regulate IGFBP-3 expression (92).

IGFBP-3 can influence proliferation, migration, and apoptosis independently of its effects on IGF signaling (93). IGFBP-3 can attenuate mitogenic signaling and proliferation by activating a phosphotyrosine phosphatase that inhibits IRS-1 and IRS-2 proteins, terminating signaling through the PI3K/Akt and MAPK/ERK1/2 pathways (94). In both prostate and ovarian carcinoma cells, IGFBP-3 was shown to have IGF-independent inhibitory effects on adhesion, migration and invasion (95;96). It has also been demonstrated that IGFBP-3 can translocate into the nucleus and interact with nuclear receptor retinoid X receptor (RXR) and NR4a1 to induce apoptosis (97;98). IGFBP-3 is also a mediator of apoptosis induced by TGF-α and TGF-β in multiple cell types. The multifunctional roles reported for IGFBP-3 are likely to be influenced by posttranslational

modifications, susceptibility to proteases and/or interactions with several signaling pathways (99-102). IGFBP-3 is secreted as a phosphoprotein and shown to be phosphorylated *in vitro* by casein kinase II (CKII), cAMP-dependent protein kinase (PKA), double-stranded DNA-dependent protein kinase (DNA-PK) as well as ERK1/2 (103). Phosphorylation of IGFBP-3 can affect its nuclear import, binding affinity to other proteins, and growth inhibitory actions.

3.6. FABP7

Fatty acid binding proteins (FABPs) are a family of small (15kDa) highly conserved proteins that bind long-chain fatty acids and other hydrophobic ligands and thus have a role in lipid metabolism. This family is divided into two subgroups, one cytoplasmic (FABPc) and one associated with the plasma membrane (FABPm). There are nine tissue-specific FABPs (FABP1 - FABP9) found in liver, intestine, heart, testis, adipose tissue, epidermis as well as brain and peripheral nervous system (104). FABP7, also known as brain lipid binding protein (BLBP), is expressed in radial glia cells during development and regulated through activation of Notch receptors (105;106).

FABPs expression in a given tissue reflects its lipid-metabolizing capacity. In cells like hepatocytes and adipocytes with high lipid biosynthesis and turnover of fatty acids, FABPs make up between 1% and 5% of all soluble cytosolic proteins (104). FABPs facilitate uptake of fatty acids by binding and minimizing the amount of unbound fatty acid in the cells thereby creating a concentration gradient. In addition, binding of fatty acids to FABPs increases their solubility and reduces potentially harmful excess of amphipathic molecules inside the cells (107).

FABPs have also been implicated in gene regulation, cell signaling, growth and differentiation and are considered as co-activators in PPAR-mediated gene control since they can enter the nucleus and target fatty acids to transcription factors (108). By binding mitogens and interacting with other proteins, FABPs are thought to contribute to growth and differentiation (109).

There is evidence that different FABPs are involved in cancer development and progression. A decrease in FABP1 level was observed with progression of colon cancer while loss of FABP4 expression is reported in bladder cancer (110;111). In contrast, both FABP1 and FABP2 are over-expressed in prostate and breast cancers (112;113). In prostate cancer FABP5 expression was associated with poor prognosis (114). Moreover, high FABP7 expression in glioblastomas is related to poor prognosis (115). FABP7 expression has also been reported in melanomas, where it is suggested to play a role in cell proliferation and

invasion (116). Recently, FABP7 was detected in patients with the basal like subtype of breast cancer and associated with better clinical outcome (117).

3.7. Melanoma

Malignant melanoma (melanoma) is derived from the pigment-producing cells termed melanocytes. These cells originate in the neural crest (ectoderm) and during embryogenesis migrate to the basal layer of the epidermis, uveal tract in the eye and mucous membranes. Although cutaneous melanoma is most frequently occurring, cancer can develop in all melanocyte containing tissues (118).

3.7.1. Epidemiology and risk factors

Even though melanoma accounts for only 4% of all dermatological cancers, it is highly aggressive and responsible for 80% of total deaths related to skin cancer (119). Each year approximately 160 000 new cases of melanoma are diagnosed and about 40 000 deaths are caused by this disease worldwide (120) (Figure 7). The highest incidence rates are found in Australia, New Zealand, North America and northern countries of Europe. With 1 200 new cases diagnosed each year, Norway is among the European countries with the highest incidence (121). Major factors that indicate a high risk of melanoma include a family history of multiple benign or atypical nevi, fair skin and excessive exposure to UV radiation, resulting in burning, mainly during childhood (122).

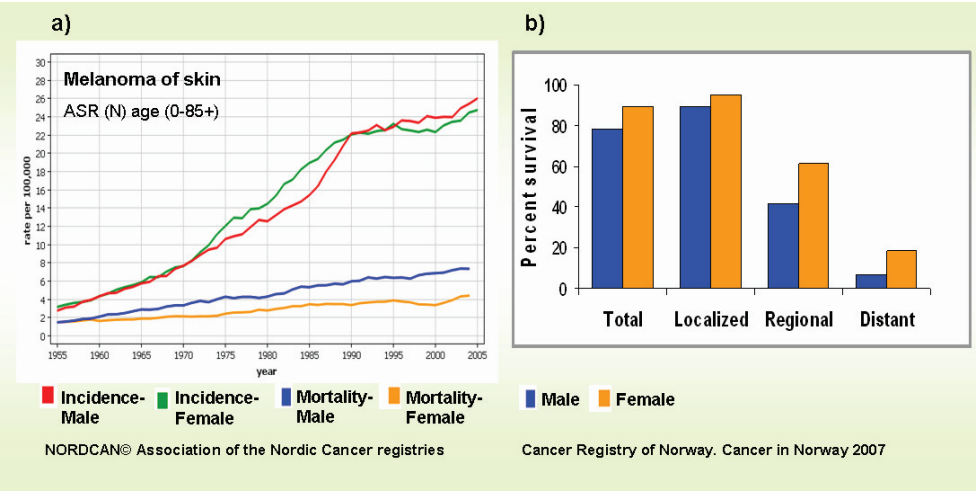

Figure 7. a) Age-standardized incidence and mortality rate for melanoma in Norway b) 5-year survival rate of patients diagnosed from 1997 to 2001 (raw data obtained from Cancer Registry of Norway 2007)

3.7.2. Melanoma stages and prognostic factors

The diagnosis of melanoma is based on several criteria and the “ABCDE" method of identification is widely used (123). This method analyzes five clinical characteristics including asymmetry (A), border irregularity (B), color variation (C), a diameter of 6 mm or more (D) and evolving (E), meaning changes in the lesion over time [4]. However, some melanomas lack all or most of the features defined in the "ABCDE" criteria (124). Further examination of the skin biopsy is, therefore, needed to determine whether the mole is benign or malignant.

Once a diagnosis has been made, the stage of the disease can be assessed by determining Breslow’s thickness of the primary lesion, ulceration and the presence of metastases (125;126). Tumors are grouped into the following main stages: Stage 0 melanoma, also called melanoma *in situ*, involves the epidermis but has not reached the underlying dermis. Stage I and II include tumors less than 1 mm thick and between 1 and 4 mm, respectively, and with no evidence of regional lymph node or distant metastases. Stage III melanomas have spread to regional lymph nodes while stage IV tumors are associated with metastases to distant sites in the body. The most common sites of metastases are skin, subcutaneous tissues, distant lymph nodes as well as lungs, liver and brain. The most powerful independent prognostic factors for stage I and II cutaneous melanoma are tumor thickness and ulceration.

If detected early, stage I melanoma is curable with surgery and 5-years survival rate is above 90% (127). Patients with advanced (stage III) and distant metastatic (stage IV) disease at presentation can expect 5-year survival rates of 60%, and 5-15%, respectively. The median survival of stage IV patients is 6-9 months (128;129).

3.7.3. Melanoma subtypes

Based on the combination of clinical and pathological features, melanomas can be divided into four main clinical subgroups. These include superficial spreading melanoma which account for 70% of all cases, nodular melanomas (15%), acral lentiginous melanoma (10%) and lentigo maligna melanoma (5%) (130). Lentigo maligna melanoma is more prevalent on chronically sun-damaged skin of head, neck, and arms in elderly. Acral lentiginous

melanoma is the most common form of melanoma in non-Caucasians, developing on palms, soles, mucous membranes and underneath or near fingernails and toenails (131). With the exception of nodular melanomas, which are more aggressive, the growth patterns of the other subtypes are characterized by an *in situ* growth phase (radial growth) that may last for months before dermal invasion occurs.

3.7.4. Melanoma progression

The skin is composed of three primary layers: epidermis, dermis and subcutis. Melanocytes reside in the basal layer of the epidermis, forming an epidermal melanin unit in contact with basal keratinocytes. This unit is characterized by a life-long stable ratio of 1:36 between melanocytes and keratinocytes (132). Homeostasis is maintained by keratinocytes regulating melanocyte division by growth factors and cell-cell adhesion molecules. This fine balance is disturbed during transformation into a nevus or a melanoma. According to Clark's model, melanomas develop and progress in a sequence of steps from normal melanocytes to metastatic melanoma via common acquired and congenital nevi without dysplasia (benign nevi), dysplastic nevi, radial-growth phase (RGP) melanoma, vertical-growth phase (VGP) melanoma and metastatic melanoma (Figure 8). However, RGP or VGP melanomas may also arise directly from melanocytes without a previous benign or borderline melanocytic lesion. Approximately 70% of the melanomas appear on clinically normal skin whereas 30% develops in association with pre-existing nevi. Transition from RGP to VGP is considered to be a critical step in progression since VGP melanomas are able to grow anchorage-independently and have acquired metastatic competence that is not present in RGP lesions (133).

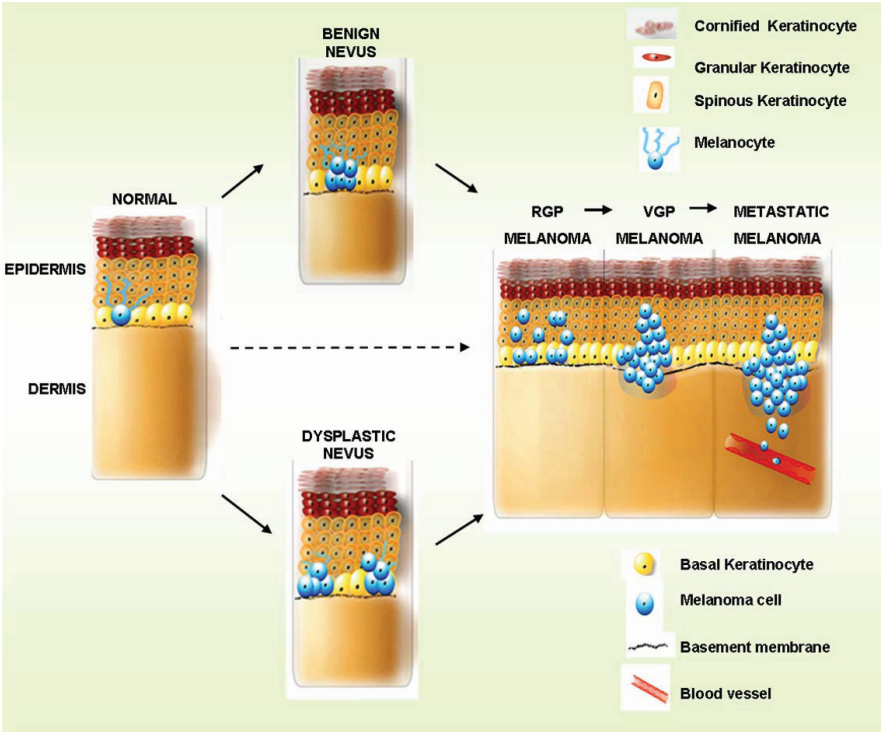

Figure 8. Development of melanoma. Adapted from ref (134).

3.7.5. Molecular profile of melanoma

Approximately 5% to 10% of all cutaneous melanomas are hereditary. In 20% to 40% of these families, germline mutations in the *CDKN2A* gene residing on chromosome fragment 9p21 have been identified (135). The *CDKN2A* gene encodes two different proteins: $p16^{INK4A}$ encoded by exons 1a, 2, and 3 and $p14^{INK4D/ARF}$ encoded by alternative splicing of an alternative exon 1b to exon 2. Both proteins have a tumor-suppressor function, regulating cell proliferation by inhibition of CDKs or by enhancing the effect of p53. In addition, 2% of the families harbor mutations in the *CDK4* gene affecting the $p16^{INK4A}$ binding site (136;137).

Hyperactivation of the MAPK/ERK1/2 pathway has a central role in the tumorigenesis of melanomas (138). The most common alterations leading to this hyperactivation are gain-of-function mutations in *NRAS* or *B-Raf*. While *NRAS* is mutated in between 4% and 50% of melanomas, 25% to 80% harbor *B-Raf* mutations (139). The most common aberration in the

NRAS gene is a substitution of glutamine to either lysine (Q61K) or arginine (Q61R) at codon 61, while mutations in *B-Raf* most often involve valine to glutamate substitution at residue 600 (V600E) (140;141). However, *B-Raf* is also mutated in between 20% and 80% of benign nevi which typically remain in a growth-arrested state for decades and only rarely progress into melanoma (140;142). Whereas B-Raf^{V600E} stimulates melanoma cell proliferation, sustained B-Raf^{V600E} expression in normal melanocytes leads to cell cycle arrest accompanied by induction of p16^{INK4A}, resulting in senescence (143-145). Induction of senescence by B-Raf^{V600E} suggests that additional genetic or epigenetic changes are required to induce full melanocyte transformation (146;147).

The PI3K/Akt signaling pathway is another important survival pathway in melanoma. PI3K mutations and activation of Akt are detected in 3% and 60% of melanomas, respectively, whereas loss of PTEN function occurs in between 5% and 20% of late-stage tumors (148-150). In melanoma subtypes where *B-Raf* and *NRAS* mutations are rare, including melanomas on mucosal membranes, acral skin, and skin with chronic sun-induced damage, amplifications and activating mutations of *C-KIT* have been observed (151). C-KIT is a RTK upstream of the PI3K/Akt and MAPK/ERK1/2 pathways.

The microphtalmia-associated transcriptional factor (MITF) is a basic helix–loop–helix leucine zipper transcription factor that regulates development, differentiation and maintenance of melanocytes, but is also essential for melanoma cell proliferation and survival (152). Recently it was shown that MITF is amplified in a small subset (10–16%) of metastatic melanomas in which *B-Raf* is mutated (153).

During progression from RGP to VGP, melanoma cells alter the expression pattern of cell surface cadherins responsible for cell-cell adhesion. Loss of E-cadherin and subsequent breakdown of melanocyte-keratinocyte interactions followed by upregulation of N-cadherin is characteristic for melanoma cells (154;155). Furthermore, loss of E-cadherin expression can also lead to increased signaling through the Wnt/β-catenin signaling pathway. Changes in components of the Wnt/β-catenin signaling pathway, promoting cell proliferation through regulation of genes like *c-Myc*, *MITF*, *CCND1* (cyclin D1) and *MMP-7*, have been reported in many cancers, including melanoma (156). Progression from RGP to VGP is also associated with increased expression of αVβ3 integrin (157). Integrins mediate contact between cells and components of the extracellular matrix like fibronectin, collagens and

laminin. Integrin αVβ3 can also stimulate the motility of melanoma cells by inducing expression of matrix metalloproteinase 2, an enzyme that degrades collagen in the basement membrane (158).

4. SUMMARY OF THE PAPERS

Paper I. *Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome*

In this study, we analyzed the protein expression of activated Akt (pAkt) and PTEN in a panel of 41 benign nevi, 162 primary (103 superficial spreading and 59 nodular) and 71 metastatic melanomas using immunohistochemistry and correlated the expression level with clinicopathological parameters. Cytoplasmic and/or nuclear expression of pAkt was seen in 54% of the nevi, 71% of the primary tumors and 72% of the metastases. Cytoplasmic PTEN staining was observed in 88% of the primary tumors and 90% of the metastases whereas no immunoreactivity was detected in benign nevi.

We found a significant correlation between PTEN and cytoplasmic pAkt expression ($P<0.001$) in primary tumors. In superficial spreading melanomas, cytoplasmic pAkt expression showed a positive association with cyclin A ($P=0.038$), which was not the case for nodular melanomas ($P=0.22$). Furthermore, we did not find any association between disease-free and overall survival and cytoplasmic pAkt and PTEN expression when performing Kaplan-Meier analysis. However, complete lack of nuclear pAkt expression was a predictor of shorter disease-free survival ($P=0.025$) for patients with superficial spreading melanomas. In conclusion, our results suggest that the PI3K/Akt pathway is activated in a relative high number of melanomas without loss of PTEN. The current model of a simple linear relationship between PTEN and activation status of Akt can not explain these results. These two factors do not appear to be valuable prognostic markers in malignant melanoma.

Paper II: *The fatty acid protein 7 (FABP7) is involved in proliferation and invasion of melanoma cells*

Using gene expression profiling (AffymetrixTM) we identified FABP7 as one of the most differentially expressed genes in melanoma cells cultivated as multicellular aggregates (spheroids) treated with the PKC activator PMA and/or the MEK1 inhibitor PD98059 (159). FABP7 mRNA and protein levels were down-regulated after treatment of melanoma cell lines with PMA and/or PD98059. *In vitro*, siRNA mediated down-regulation of FABP7 protein led to decreased cell proliferation and invasion, but did not affect apoptosis.

Immunohistochemical staining of 11 benign nevi, 149 primary (93 superficial spreading and 56 nodular) and 68 metastatic melanomas reveald that 91% of the nevi, 71% of the

primary and 70% of the metastases expressed FABP7 in the cytoplasm and/or the nucleus In superficial spreading melanomas, FABP7 expression was associated with tumor thickness (P = 0.021). Furthermore, there was a trend for an association between FABP7 expression and Ki-67 score (P = 0.070) and shorter relapse-free survival (P = 0.069) in this group of patients. Taken together, these results suggest that FABP7 is a downstream target of both PKC and the MAPK/ERK1/2 pathway in melanoma cells. FABP7 does not seem to be involved in apoptosis in melanoma cells, but rather contributes to proliferation and invasion.

Paper III: *Biological effects induced by insulin-like growth factor binding protein 3 (IGFBP-3) in malignant melanoma.*

In this paper we have characterized expression, function and regulation of IGFBP-3 in melanomas as well as evaluated its potential as a biomarker. IGFBP-3 showed variable expression in human melanoma cell lines and no clear differences were observed between cell lines originating from primary tumors vs. metastases.

Reintroduction of the protein in cells naturally lacking IGFBP-3 led to induction of apoptosis. In cell lines constitutively expressing IGFBP-3, siRNA mediated silencing led to a cell line dependent decrease in proliferation, but had no effect on apoptosis and invasion. In patient material, we found that IGFBP-3 was not expressed in benign nevi while a slight increase in protein expression was seen in primary and metastatic tumors. However, overall expression of the protein was low and we found no correlation between disease stage and circulating levels of IGFBP-3 in serum. For this reason, we concluded that IGFBP-3 has limited potential as a predictive biomarker in melanoma. We detected IGFBP-3 promoter methylation in both melanoma cell lines and patient material suggesting that IGFBP-3 is regulated by epigenetic silencing. In addition, we found that both the PI3K/Akt and the MAPK/ERK1/2 pathways can regulate IGFBP-3 expression level. In summary, our findings suggest that IGFBP-3 can have several roles in melanomas, influencing both apoptosis and proliferation. Development of resistance to the antiproliferative effects of IGFBP-3 may be an important step in progression of this disease.

5. METHODOLOGICAL CONSIDERATIONS

5.1. Tissue Material

The tissue material used in this thesis was selected from a malignant melanoma archive at the Norwegian Radium Hospital and is derived from patients that underwent surgery since the 1980s. Research on this material has been approved by The Regional Committee for Medical Research Ethics in Norway.

5.2. *In vitro* Cell Cultures

Use of *in vitro* cell cultures for studies of basic biological mechanisms in both normal and cancer cells has many advantages. However, it is also important to recognize the limitation of these models when interpreting the results. Continuous cell cultures are poorly differentiated, and lose many of the phenotypic characteristics of the original cell type *in vivo*. Heterogeneity in growth rate and the capacity to differentiate within the population can produce variability and there is evidence that cell lines at high passage numbers show changes in morphology, growth rates as well as response to stimuli and protein expression, compared to lower passage cells (160;161). Lack of standardized culturing conditions can result in different phenotypes being expressed by the same cell lines, making the interlaboratory comparability of *in vitro* results difficult. To minimize these variations, *in vitro* cell cultures used in this study were routinely sustained in RPMI 1640 medium supplemented with 5%-10% fetal calf serum (FCS) and experiments were performed under minimal changes to these conditions. Furthermore, we have avoided using cell lines that have been kept in culture too long.

5.3. Measurements of cell viability and apoptosis

In this thesis we aimed to study biological processes affected by particular signaling pathways or treatments applied, and for this reason we have used several methods to measure cell viability, proliferation and apoptosis. Cell viability was measured using the MTS-assay (Cell Titer Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) which is a colorimetric method for detecting the amount of living cells. The colored end product is linearly correlated to the dehydrogenase activity in metabolically

active cells. However, this method can poorly distinguish between cell cycle arrest and apoptosis. Therefore, we have also used the [3H]-thymidine incorporation assay for DNA synthesis to measure proliferation rates.

The TUNEL (terminal deoxynucleotidyl transferase end labeling) method was used to evaluate the degree of apoptosis. This method is based on detection of internucleosomal DNA fragmentation, a typical event during apoptosis. The formation of DNA strand breaks is detected by enzymatic labeling of the 3'-OH termini with modified nucleotides. However, in some cases, internucleosomal DNA degradation does not occur during apoptosis, making the number of DNA strand breaks inadequate to distinguish apoptotic cells by this method (162;163). Likewise, DNA fragmentation can also stop after the initial cleavage of DNA to fragments of 50 to 300 kb, resulting in low frequency of DNA strand breaks (164). In addition, TUNEL will stain necrotic cells to some degree due to extensive DNA degradation. Permeabilization and the subsequent cell washing after ethanol fixation, allows extraction of small pieces of fragmented DNA, leading to a diminished number of DNA strand breaks.

5.4. Protein Detection

We have used both immunohistochemistry and Western blot analysis to study expression of proteins of interest in patient material and cell lines, respectively. Immunohistochemistry identify proteins by a microscopically evaluation of tissue sections. This method provides information about the localization and distribution of a particular protein in the entire tissue section as well as in single cells. Specificity and amount of antibody used, pretreatment such as fixation and antigen retrieval and the use of different controls, in addition to subjectivity of interpretation of staining patterns and different scoring systems may influence the results (165). All antibodies used in this thesis were commercially available. For antibodies that could be used in both applications, the specificity was tested by western blot analysis to confirm that the band of predicted size is detected. The immunohistochemical method was optimized for each antibody and all series included positive control samples which were either a tissue known to contain the antigen under study or cell lines examined by western blot analysis. Negative controls included substitution of the monoclonal primary antibody with mouse myeloma protein of the same subclass and concentration and replacement of the

polyclonal antibody with normal rabbit IgG of the same concentration as the polyclonal antibody In addition, when possible, we used cell lines transfected with siRNA, targeting the protein of interest, as negative controls. All controls gave satisfactory results.

5.5. Cell Migration Assay

In papers II and III we used Matrigel invasion chambers (BD Biosciences, San Jose, CA) to study cell invasion. Important considerations when performing these experiments had to be taken into account, including how to quantify the number of migrating cells and which cells to include. Moreover, we had to consider whether the treatment applied had additional effects on the tumor cells which could affect the measurement. Such experiments are technically demanding and had to be repeated many times to obtain satisfactory reproducibility.

One of the critical factors is thickness of the Matrigel in each well. Even though we used pre-coated commercial chambers, we did experience variation between batches. For this reason, the results from the experiments were presented as percentage compared to the adequate controls. Since the WM35 cell line is poorly aggressive, relatively few cells invaded through the Matrigel after 24 hours of incubation. Due to this, we chose to extend the incubation time to 48 hours. In paper II we quantified invading cells at the bottom surface of the chamber membranes by fixing, staining with hematoxylin and counting as recommended by the supplier. However, in this case it was difficult to exclude effects of cell viability on the results. In order to avoid similar problems in paper III, we chose to incorporate [3H]-thymidine in the cells prior to seeding and quantified the number of cells in both compartments (upper and lower side of the membrane) separately using a scintillation counter. By using the ratio between cells numbers in both compartments we could more accurately calculate the percentage of invading cells and eliminate the impact of seeding and viability variability.

5.6. Qunatitative Real-Time RT-PCR

When performing quantitative PCR experiments, it is of importance to minimize errors that can be introduced due to differences in starting amount of RNA as well as differences in efficiency of cDNA synthesis or PCR amplification. This and sample variation is corrected for by the use of control genes, which are often referred to as housekeeping genes. However, the expression level of these genes may vary among tissues or cells and may change under certain circumstances. Thus, the selection of housekeeping genes is critical for gene expression studies. In our studies we have used the beta-glucuronidase (GUS) gene to normalize the mRNA expression level of genes of interest. GUS was chosen based on our pilot studies using TaqMan® Low Density Array Human Endogenous Control Panel (Applied Biosystems) which contains commonly used housekeeping genes that exhibit minimal differential expression across 16 different tissues. GUS was shown to have stable transcript level under the various experimental conditions.

5.7. siRNA transfection

Through this thesis we utilized siRNA to transiently down-regulate proteins in order to study their contribution to tumorigenesis of melanomas. All siRNA oligonucleotide duplexes (Stealth RNAi) were obtained from Invitrogen (Invitrogen, San Diego, CA). Negative controls included siRNA with the same nucleotide composition as the target siRNA, but lacking significant sequence homology to the genome (scrambled siRNA). To verify siRNA mediated knock-down of proteins, Western blot analysis was performed for each experiment.

The use of siRNA can induce cellular stress response pathways such as the interferon response. This can result in growth inhibition and cellular toxicity, making it difficult to assess whether the observed cellular effects are due to non-specific stress responses or to loss of function of a target gene. All our transfections were optimized to achieve the greatest amount of target-specific knock-down of expression, as well as a low rate of toxicity in cells transfected with scrambled siRNA by using the lowest effective concentrations. However, the possibility that the observed phenotype might be due to knock-down of other genes caused by nucleotide sequence similarity between the siRNA molecule and short motifs in their mRNAs can not be completely excluded

6. RESULTS AND DISCUSSION

6.1. Activation status of the PI3K/Akt pathway and its association with clinical outcome in melanoma patients

During the last decade the PI3K/Akt and MAPK/ERK1/2 pathways have emerged as central signaling cascades deregulated in melanoma, contributing to both development and progression of the disease (166). In this context our project has focused on validation of these pathways in clinical settings and identification and characterization of new downstream targets.

The first evidence of deregulated PI3K/Akt pathway came in 1988, when Parmiter et al. reported loss of chromosome 10, harboring the PTEN gene, in melanoma samples (167). However, before 2005, few studies had evaluated the role of altered PTEN expression or activation of Akt in relation to clinical outcome for melanoma patients. Thus, in paper I we analyzed the expression of PTEN and activation status of Akt in melanocytic tissues using immunohistochemistry and correlated our findings to clinical parameters.

We observed variable degree of PTEN cytoplasmic staining in 88% of primary and 90% of metastatic melanomas, suggesting that complete lack of PTEN protein expression is not a frequent event in melanomas. Notably, however, approximately 30% of both primary and metastatic tumors expressed PTEN in less than 50% of the cells. In support of our results, Zhou *et al.* reported lack of PTEN in 15% (5/34) and low expression in 50% (17/34) of the tested melanomas. In addition, others have reported lack of or decreased PTEN protein expression in up to 30-40% of melanomas (168-172). Even though mutations in *PTEN* are not frequent in melanomas (173;174), it is assumed that PTEN can be disrupted in as many as 40–50% of sporadic melanomas by other mechanisms, such as epigenetic silencing (175), altered subcellular localization (169) or ubiquitination (176).

Although the frequency of PTEN loss in our primary melanoma samples was in accordance with other observations, total lack of immunoreactivity in benign nevi was highly surprising and in contrast to previous reports (171). However, loss of PTEN protein expression in nevi has also been reported in a study by Packer *et al.* (172) where 59% nevi showed little or no PTEN staining while it was detected in 67% of primary and 37% of metastatic melanomas.

Thus, based solely on these immunohistochemical results, it is difficult to draw conclusions on the role of PTEN in benign nevi.

Lack of immunoreactivity does not necessarily reflect absence of protein, but can rather be due to methodological factors like suboptimal tissue preparation and inadequate epitope unmasking. In addition, the use of different antibodies is likely to result in different PTEN staining which could partially explain the discrepancy in reported results. Interestingly, while our paper was under revision, Pallares *et al.* published results describing PTEN staining pattern in a panel of endometrial carcinomas, using four different anti-PTEN commercial antibodies, including the polyclonal antibody from Zymed used in our study (177). In addition, they also correlated the results to the presence of abnormalities in the *PTEN* gene and expression of phosphorylated Akt (pAkt). In accordance with our observations, they reported cytoplasmic PTEN staining using the Zymed antibody. However, a wide variability in the results was obtained using the different antibodies. Furthermore, the Zymed PTEN antibody showed no correlation with pAkt immunostaining. Surprisingly, when correlated with the presence of molecular alterations in the *PTEN* gene (mutations, loss of heterozygosity, or promoter hypermethylation), the mean H-score was higher in tumors with molecular alterations of *PTEN* as compared to those having wild type *PTEN*.

Although previous studies have suggested that loss of PTEN expression is associated with poor prognosis in other tumor types (178-180), we did not find any association with clinical parameters. This is in agreement with the study by Whiteman *et al.* (169), who failed to demonstrate any association between PTEN expression and clinical features such as Clark's level and Breslow thickness. Furthermore, Mikhail *et al.* (170), found no correlation between PTEN expression and disease-free and overall survival in melanoma patients. Together, these results suggest that the tumor expression level of PTEN by it self has limited utility in predicting clinical outcome for melanoma patients. Nevertheless, the importance of PTEN has been confirmed by many functional studies and recently Dankort *et al.* showed that PTEN loss and B-Raf^{V600E} cooperate to promote metastasis in melanoma (181). In their mouse model, expression of B-Raf^{V600E} at physiological levels, in combination with PTEN loss, led to tumor development.

When examining Akt activation in our melanoma panel, we observed variable degree of cytoplasmic and nuclear pAkt staining in 44% of the benign nevi and in 68% of the melanomas. This was in accordance with a study by Dhawan *et al.* who reported little pAkt immunoreactivity in benign nevi but high pAkt levels in up to 66% of dysplastic nevi and melanomas (182). Similarly, Stahl *et al.* showed that selective activation of the Akt3 isoform, caused by increased gene copy number and decreased PTEN protein activity, occurs in 43–60% of sporadic melanomas (149). In their study, weak or moderate pAkt staining was detected in 100% of common nevi, while strong staining was observed in 12% of dysplastic nevi, 53% of primary and 67% of metastatic melanomas. Furthermore, simultaneously with us, Dai *et al.* reported strong pAkt expression in 17%, 43%, 49% and 77% of normal nevi, dysplastic nevi, primary and metastatic melanomas, respectively (183). Collectively, these studies have confirmed that activation of the PI3K/Akt pathway is a frequent event in melanomas.

Dai *et al.* also found that pAkt expression was inversely correlated with both overall and disease-free survival, and was a poor prognostic factor for patients with melanomas less than 1.5 mm in thickness. We did not observe any associations with disease-free or overall survival in our cohort. This discrepancy might be due to different evaluation of immunostaining, definition of low vs. high Akt activation, subclassification of our tumors as well as subdividing into groups based on tumor thickness. However, we did observe a positive correlation between cytoplasmic pAkt and cyclin A, in accordance with a previous report by Shen *et al.* (184). Since cyclin A expression has been related both to proliferation as well as tumor thickness and clinical outcome in melanomas (185), activation of Akt might indirectly contribute to disease progression by increasing proliferation.

Surprisingly, in our study, lack of pAkt expression in the nucleus was a predictor of shorter disease-free survival in patients with superficial spreading melanoma. Recently, similar results were obtained by Jovanovic *et al.* (186). In further support of our findings, other studies have reported that nuclear pAkt expression is correlated with better prognosis in lung, endometrial and renal cell carcinoma (187-189). In prostate cancer, Page *et al.* reported that nuclear Akt1 and Akt2 expression correlated with parameters of favorable outcome (190). Although Akt3 is the predominant isoform activated in melanomas, specific effects of the other isoforms are not well studied. The antibody used in our study is reactive to Akt1 phospho-epitopes, but cross-reacts also with the homologous phospho-epitopes in

Akt2 and Akt3. For this reason, our results might reflect the possibility that each Akt isoform have a different role and that their subcellular localization may be important in determining cellular effects.

In contrast to previous reports, we detected a positive correlation between cytoplasmic pAkt and PTEN expression (191;192). However, similar results have been reported in a large cohort of breast and ovarian carcinomas, suggesting that a common linear model of Akt activation upon PTEN inactivation is oversimplified (193;194). In support of this view, Gewinner *et al.* recently identified inositol polyphosphate 4-phosphatase type II (INPP4B) as a new tumor-suppressor in human epithelial cells that decreases Akt activation by hydrolyzing phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) (195).

In our study, we observed a positive association between Akt and ERK1/2 activations. Since these pathways are often activated in the same cells, the observed positive correlation might reflect their simultaneous parallel activation. Still, there is also evidence that the PI3K/Akt and MAPK/ERK1/2 cascades are interconnected (196-198). The PI3K/Akt signaling pathway has in several cases been reported to inhibit, rather than increase, ERK1/2 activation. In normal cells, pAkt has been shown to phosphorylate B-Raf to decrease its activity (199;200). Recently, Cheung *et al.* suggested that B-Raf^{V600E} and Akt3 cooperatively promote melanoma development (201). They showed that activating *B-Raf*^{V600E} mutation initially promotes nevi development, but the resulting high, intense activation of the MAPK/ERK1/2 pathway inhibits further tumor progression. For further progression to occur, activation of Akt3 is required to phosphorylate and inhibit B-Raf^{V600E}, lowering the levels of the MAPK/ERK1/2 pathway activity to levels promoting, rather than retarding, melanocytic cell growth and transformation.

6.2. Identification and characterization of novel targets of the PI3K/Akt and MAPK/ERK1/2 pathways

Previously, we and others showed that the PI3K/Akt and MAPK/ERK1/2 pathways play a role in preventing anoikis and facilitating anchorage-independent growth, thereby being involved in metastasis (159;202-204). Our results showed that in the early stage melanoma cell line, WM35, which undergoes spontaneous cell death when cultivated as three-dimentional multicellular aggregates (spheroids) in suspension, activation of PKC confers

anoikis resistance partially through the MAPK/ERK1/2 pathway (159). Interestingly, in these cells, cultivation as spheroids also increased the PTEN protein level, suggesting involvement of the PI3K/Akt pathway (unpublished results). Furthermore, in addition to increasing the degree of anoikis in these cells, inhibition of the MAPK/ERK1/2 pathway using the MEK1 inhibitor PD98059 led to further increase in PTEN protein level ((159) and unpublished results).

In our further studies we exploited the spheroid model to study how these pathways contribute to survival and apoptosis/anoikis of melanoma cells and to identify and characterize novel downstream targets. We used high throughput gene expression profiling (AffymetrixTM) to identify differentially expressed genes in untreated WM35 cells cultured as monolayer and spheroids, as well as in spheroids treated with the PKC activator PMA and/or the MEK1 inhibitor PD98059. The data analysis of the microarray experiments revealed that *FABP7* and *IGFBP-3* were among the most significantly differentially expressed genes (unpublished results). For this reason we have in paper II and III further characterized their role in melanoma biology.

6.2.1. FABP7

Our data analysis showed that both PKC activation and MEK1 inhibition, previously reported to exert opposite effect on anchorage-independent survival (159), led to down-regulation of FABP7 mRNA in WM35 spheroids, arguing against its involvement in promotion of anchorage-independent survival. Activation of PKC resulted in down-regulation of FABP7 even in the presence of activated ERK1/2, suggesting that this effect is mediated independently of the MAPK/ERK1/2 pathway. Previously it has been shown that both PKC and the MAPK/ERK1/2 pathway can regulate the activity of the peroxisome proliferator-activated receptors α/γ (PPAR α/γ) (205-207). Furthermore, binding of PPAR α/γ to the response element PPRE can regulate several proteins of the FABP family, making it likely that also FABP7 might be regulated through this mechanism. (208;209). Even though we have not investigated a possible regulation of FABP7 by the PI3K/Akt pathway in paper II, our recent preliminary experiments have shown that treatment of WM35 cells with the PI3K inhibitor LY294002 leads to a decrease in the FABP7 protein expression (unpublished results). Interestingly, FABP7 has been identified as a direct target of Notch signaling in radial glial cells, which share many biological properties with melanocytes due

to their common origin (210). In melanoma cells, hyperactivation of the PI3K/Akt pathway was shown to up-regulate Notch expression (211). Thus, regulation of FABP7 via Notch and the PI3K/Akt pathway might also be possible in melanoma, but remains to be further investigated.

To clarify the role of FABP7 in the biology of melanomas, we transiently down-regulated its expression in the primary WM35 and the metastatic WM239 cell lines. This down-regulation significantly inhibited proliferation in both cell lines. However, we did not observe any effect on apoptosis. This, together with our observations in WM35 spheroids, showing that FABP7 did not contribute to anchorage-independent survival, suggests that FABP7 rather contributes to melanoma proliferation. In support of these results, Goto *et al*. showed that proliferation of melanoma cell lines is reduced upon down-regulation of FABP7 without affecting apoptosis (116). Furthermore, we showed that FABP7 down-regulation reduced the invasive potential, which is also in agreement with Goto *et al.* (116). Previously, Mita *et al.* reported that introduction of FABP7 into a FABP7-negative malignant glioma cell line enhanced its migratory properties while the opposite was observed after reduction of its expression in FABP7-positive cells (212). Altogether, our results have placed FABP7 as a possible mediator of proliferative and invasive effects downstream of PKC and the MAPK/ERK1/2 pathway in melanoma cells *in vitro*. Furthermore, regulation of FABP7 expression by the PI3K/Akt pathway is likely to occur as well.

When investigating FABP7 protein expression *in vivo*, we surprisingly observed that benign nevi had the highest protein expression, while it was slightly lower in primary and metastatic melanomas. This is in accordance with the study by de Wit *et al.* who, found that the FABP7 was down-regulated in melanoma tissue compared to nevi (213). However, our analysis of the clinical data revealed that in superficial spreading melanoma, high FABP7 expression is positively associated with Ki-67 expression and thicker primary tumors. Although, not reaching significance, probably due to limited sample size, an association between FABP7 expression and shorter disease-free survival was observed in this group of patients as well, suggesting that FABP7 may contribute to disease progression by increasing cell proliferation *in vivo*.

Taking into consideration both the *in vitro* results and the correlation of FABP7 expression with the disease progression, high expression of FABP7 in nevi compared to melanomas seems contradictory. Interestingly, in the study by Goto *et al.*, FABP7 expression decreased with disease progression due to genomic instability and LOH of the 6q21–23 chromosome region containing the *FABP7* gene (214). As cutaneous melanoma progress, genomic instability becomes more prominent and LOH becomes more frequent in specific chromosome regions (215). Furthermore, the majority of benign nevi are terminal lesions that do not progress to melanoma and the molecular events in these cells differ from those in melanoma cells. For this reason it is possible that FABP7 have a different role in nevi.

Taken together, our *in vitro* and *in vivo* observations support the hypothesis that FABP7 has disease promoting features in melanomas. In accordance with this, Goto *et al.* reported that FABP7 expression in melanoma metastasis was associated with poor clinical outcome (214). Moreover, a negative association between FABP7 expression and poor prognosis was previously observed in glioblastoma patients (115;216). However, FABP7 expression has also been found in the mammary gland, but in a mouse breast cancer model its over-expression inhibited tumour growth (217;218). Recently, Zhang *et al.* reported that in the basal-like subclass of breast cancer, FABP7 expression was associated with better patient outcome, suggesting that FABP7 effects are tissue specific (117).

6.2.2. IGFBP-3

As previously described, IGFBP-3 was another gene found to be highly differentially expressed in our microarray studies. Data analysis showed that treatment of WM35 spheroids with the MEK1 inhibitor PD98059 led to decreased IGFBP-3 mRNA levels placing it downstream the MAPK/ERK1/2 pathway. Previously, several studies have suggested involvement of IGFBP-3 in different cancer types (219-221). However, only one study by Xi *et al.* has described its potential role in melanomas, suggesting that IGFBP-3 expression may be an important migration and proliferation factor necessary for metastasis (222).

Validation of our microarray data revealed that although highly differentially regulated upon PD98059 treatment, mRNA and protein levels of IGFBP-3 in WM35 cells were very low. In

paper III we indeed showed that IGFBP-3 protein is not ubiquitously expressed in melanoma cell lines or in patient material. We found that this lack of constitutive expression could partially be explained by promoter methylation and gene silencing of *IGFBP-3*, as previously reported in several other tumor types (223-225). Evidence of promoter methylation and gene silencing accompanied with low protein expression, argue against the proposed disease promoting role in melanoma.

To clarify these discrepancies, we chose to re-express IGFBP-3 protein in two IGFBP-3 negative cell lines and transiently down-regulate its expression in two IGFBP-3 positive cell lines. We observed that re-expression of IGFBP-3 induced apoptosis, which is in agreement with previous studies showing that IGFBP-3 is capable of exerting pro-apoptotic effects (226;227). Also, exogenously added IGFBP-3 has been shown to induce apoptosis in several cell systems. However, in our study, exogenously applied IGFBP-3 did not exert such effects, suggesting that only intracellular IGFBP-3 has apoptosis-inducing capabilities (228;229). In support of our results, intracellular IGFBP-3 has been shown to induce apoptosis by several mechanisms including increasing the ratio of pro-apoptotic to anti-apoptotic proteins or by interacting with these, and by interacting with the retinoid X receptor (RXR) and the orphan nuclear receptor NR4a1 (98;230). Lack of effect by exogenously applied IGFBP-3 might suggest that the protein is not internalized by the cells used in our study.

In contrast to these observations, transient down-regulation of IGFBP-3 in cell lines with high endogenous IGFBP-3 expression led to cell line-dependent effect on proliferation, but did not affect apoptosis or invasion. Possibly, in these cell lines, down-regulation of IGFBP-3 leads to lower recruitment of IGF-1, and thereby reduced receptor activation, ultimately leading to decreased proliferation. However, since IGF-1 does not have a predominant role in proliferation of metastatic melanoma cells (231) and our findings demonstrate low levels of IGF-R1 in these cells, it is likely that the observed IGFBP-3 effects are IGF-1 and IGF-R1 independent. Previous studies have suggested that IGF-1 independent actions of IGFBP-3 might be mediated by its high affinity binding to putative IGFBP-3 cell surface receptors, but these have yet to be confirmed (232;233).

Taken together, these results suggest that IGFBP-3 can exert opposing cell type specific effects. In support of our results, a recent study by Dupart *et al.* obtained similar results in gastrointestinal stromal tumor cell lines (234). In addition, McCaig *et al.* showed that IGFBP-3 normally induced apoptosis in breast Hs578T cancer cells, but this effect was reversed in non-IGF-responsive Hs578T cells when plated onto fibronectin (235). Furthermore, they showed that IGFBP-3 could have positive or negative effects on growth and survival in these cells, depending on the status of cholesterol-stabilized integrin receptor complexes (236).

In an attempt to explain different expression patterns and the differences in biological effects exerted by IGFBP-3, we further analyzed downstream components of the IGF signaling pathway. The results revealed PTEN loss in IGFBP-3 expressing cell lines and constitutive activation of the MAPK/ERK1/2 and PI3K/Akt pathways. Treatment of the cells with the MEK1 inhibitor PD98059 or the PI3K inhibitor LY294002 led to transcriptional down-regulation of IGFBP-3, suggesting that IGFBP-3 can be regulated through both the MAPK/ERK1/2 and PI3K/Akt pathways. Furthermore, down-regulation of PTEN protein in unmethylated IGFBP-3 negative melanoma cell line with wild type *PTEN*, increased IGFBP-3 protein expression, again suggesting that PTEN can regulate IGFBP-3 either directly or via the PI3K/Akt pathway. Regulation of IGFBP-3 by the MAPK/ERK1/2 and PI3K/Akt pathways has previously been shown in other cell types, but appears to be cell type specific. Thus, in a study of gastric cancer cell lines, Yi *et al.* showed that inhibition of the PI3K/Akt pathway led to up-regulation rather that down-regulation of IGFBP-3 (237).
Taken together, these observations made us hypothesize that in melanoma cell lines lacking high constitutive activation of the MAPK/ERK1/2 and PI3K/Akt pathways, IGFBP-3 suppress tumorigenesis by inducing apoptosis. Activation of these pathways during disease progression, however, allows cells to escape from the growth-inhibitory effects of IGFBP-3 and rather utilize it to stimulate growth and proliferation.

The *in vitro* observations were also confirmed in our in vivo studies. We found that IGFBP-3 protein is not highly expressed in melanoma biopsies. As seen *in vitro*, lack of protein expression in melanoma biopsies could be explained by methylation of the IGFBP-3 promoter. Furthermore, a trend towards an inverse association between IGFBP-3 and PTEN protein expression was observed in clinical samples, suggesting that regulation via the PI3K/Akt signaling pathway might also occur *in vivo*. Nevertheless, although overall low,

IGFBP-3 expression was up-regulated in metastatic lesions (37%) as compared to primary melanomas (6%) and benign nevi (0%) which is in agreement with our observations in the cell lines and the study by Xi *et al*. (222). Induction of IGFBP-3 mRNA in response to hypoxia in lung carcinoma cells has been shown to be mediated by both p53-independent and -dependent mechanisms (238). It is possible that in fast growing metastatic melanoma tissues, hypoxic areas occur more frequently than in smaller primary tumors, which could partially contribute to induction of IGFBP-3.

While high IGFBP-3 expression has been associated with unfavorable prognosis in breast cancer patients (239), elevated serum IGFBP-3 levels were reported to reduce the relative risk of developing breast cancer (240). In our study, we did not reveal any differences in IGFBP-3 levels in the plasma samples from normal control individuals compared to melanoma patients with stage II and IV disease. The fact that in most of the biopsies, IGFBP-3 expression was seen in less than 5% of the tumor cells, may explain the lack of correlation between tumor progression and IGFBP-3serum levels, implicating that IGFBP-3 can not be used as a serum marker in patients with melanoma. In support of these results, studies in other cancer forms found no predictive value for serum IGFBP-3 levels (220;241). Recently Yu *et al.* showed that IGFBP-3 serum levels in melanoma patients and healthy adults were comparable and thus had no clinicopathological relevance (242).

7. CONCLUDING REMARKS

In this thesis we have focused on studying how the PI3K/Akt and MAPK/ERK1/2 pathways contribute to development and progression of melanoma. We confirmed that the PI3K/Akt pathway is deregulated *in vivo* since both loss of PTEN expression and high activation of Akt occur in melanoma lesions. The presence of pAkt in benign nevi suggests that activation of the PI3K/Akt pathway might be an early event, possibly contributing to tumor development.

In superficial spreading melanomas, activated cytoplasmic Akt is positively associated with cyclin A, through which it might influence cell cycle progression. However, nuclear pAkt is associated with longer disease-free survival in these patients, suggesting that activated Akt exerts different functions in melanomas depending on its localization. Nevertheless, lack of a direct association between PTEN expression or Akt activation and patient outcome make these factors less useful as prognostic biomarkers.

Furthermore, we have identified two proteins, FABP7 and IGFBP-3, as downstream targets of the PI3K/Akt and MAPK/ERK1/2 pathways in melanoma cells. We showed that FABP7 is involved in proliferation and invasion *in vitro*. FABP7 is expressed in melanocytic lesions and associated with proliferation and tumor thickness in patients with superficial spreading tumors, suggesting that for these patients, FABP7 could be a potential target for therapy.

IGFBP-3 can exert dual biological functions in melanomas by either inducing apoptosis in early stages of the disease, or positively contributing to proliferation in later stages. During disease progression, further accumulation of malignant changes in the cells, including increased activation of the PI3K/Akt and MAPK/ERK1/2 pathways, makes cells refractory to the inhibiting effects of IGFBP-3 and even able to utilize it to their advantage. IGFBP-3 increases during melanoma progression, but due to its overall low expression, it has little diagnostic value as a biomarker in this cancer type.

8. FUTURE PERSPECTIVES

Even though collectively, PTEN deregulation occurs in 30% to 40% of melanomas, the frequency of Akt activaton appears to be even higher, suggesting that a linear relationship between these events is oversimplified. Identification of INPP4B as a new tumor-suppressor, whose down-regulation leads to enhanced Akt activation and anchorage-independent growth, adds new insight into the regulation to this pathway (195). In the study by Gewinner *et al*., deletions of chromosome region 4q31.1-3, harboring the INPP4B gene, were detected in 21.6% of the examined melanomas (195). For this reason it is of interest to investigate if INPP4B can contribute to regulation of Akt activation in melanoma cells as well. Our preliminary data suggests that INPP4B mRNA level is reduced in metastatic melanoma cell lines compared to cell lines derived from early lesions, supporting the tumor-suppressor hypothesis. Interestingly, INPP4B also emerged as one of the up-regulated genes in WM35 spheroids compared to monolayer control in our microarray study. Our further aim is to analyze INPP4B protein expression both *in vitro* and *in vivo*, and to correlate its expression to Akt activation. Our preliminary data show that down-regulation of PTEN in WM35 cells do not activate Akt significantly, even after stimulation with growth factors. We wish to examine if simultaneous down-regulation of both PTEN and INPP4B will increase Akt activation and how this will effect anchorage-independent survival and growth.

Several studies have provided evidence that as melanomas progress, there is functional redundancy between signaling pathways mediating survival. For this reason, probably only simultaneous inhibition of multiple pathways will result in synergistic induction of cell death and overcome therapy resistance. Inhibiting MAPK/ERK1/2 in conjunction with the PI3K/Akt pathway is likely to synergize at the level of Bcl-2 family proteins to induce apoptosis. Recently, the orphan nuclear receptor NR4a1 (Nur77/TR3) was identified as a possible downstream target of both of these pathways (243). Upon receiving a proper apoptotic signal, NR4a1 is phosphorylated and translocated from the nucleus to the mitochondria, where it interacts with Bcl-2 to trigger apoptosis (244). It has been shown that NR4a1 nuclear export requires prolonged MEKK1/JNK/SAPK activation and inhibition of Akt activation (245). Our microarray and RT-PCR results showed that NR4a1 is regulated by PKC and the MAPK/ERK1/2 pathway in melanoma cells. One previous study have suggested that NR4a1 is involved in apoptosis in melanoma cells (246). Interestingly, in prostate cancer, IGFBP-3 regulates translocation of NR4a1 to the mitochondria. In this

setting, NR4a1 has an important role as a mediator of IGFBP-3 induced apoptosis (98). We aim to further investigate the role of NR4a1 in melanoma proliferation and apoptosis *in vitro* and *in vivo*. We also wish to map in detail the signaling pathways involved in regulation of NR4a1 expression and translocation. Since we have shown that IGFBP-3 can induce apoptosis in melanoma cells, it will be of interest to examine if NR4a1 is involved in this process.

Two compounds shown to induce nuclear export of NR4a1 are anisomycin and CD437 (243). Anisomycin is an antibiotic which inhibits protein synthesis and activates stress-activated protein kinases JNK/SAPK and p38-MAPK. CD437 is a synthetic retinoic acid with selective apoptotic activity in a large variety of leukemic and solid tumor cells (247). Both compounds have been shown to induce apoptosis in melanoma cells, making them interesting from a clinical perspective (248;249). We wish to investigate if NR4a1 is involved in induction of apoptosis after anisomysin/CD437 treatment and explore which signaling pathways are mediating this effect.

In order to further study the contribution of the PI3K/Akt and MAPK/ERK1/2 pathways to survival of melanoma cells under anchorage-deprived conditions, we have recently preformed gene expression profiling of anoikis-sensitive WM35 cells and the anoikis-resistant variant WM35sph6, both cultured as monolayer and spheroids (unpublished results). WM35sph6 cells represent a subpopulation of WM35 cells selected for ability to grow anchorage-independently by repeated prolonged cultivation as spheroids.

Among the genes found to be differentially expressed between these two cell lines was IGFBP-7. By high affinity binding to insulin, IGFBP-7 can modify its distribution and ability to bind to the receptor, thereby influencing downstream the PI3K/Akt and MAPK/ERK1/2 pathways. Previously it has been suggested that IGFBP7 function a as tumor-suppressor in colorectal adenocarcinoma (250). More recently, Wajapeyee *et al.* suggested that B-Raf^{V600E} expression in primary melanocytic cells leads to synthesis and secretion of IGFBP-7, which acts through autocrine/paracrine pathways to inhibit MAPK/ERK1/2 signaling and induce senescence and apoptosis, thereby suppressing melanoma development (251). The authors also suggested a potential therapeutic application of these findings, since treatment of B-Raf^{V600E} melanoma cells with

recombinant IGFBP-7 was able to trigger apoptosis both *in vitro* and *in vivo*. However, the role of IGFBP-7 in melanomas is highly debated since a newly published paper by Schrama *et al.* failed to find any correlation between IGFBP-7 expression and *B-Raf* mutational status (252). Our microarray data showed that IGFBP-7 is up-regulated in the anoikis-resistant and more aggressive WM35sph6 cells, which argues against its tumor-suppressor role. It will be of interest to further characterize the role of IGFBP-7 in melanomas and its possible influence on the PI3K/Akt and MAPK/ERK1/2 pathways.

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PAPER I

Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome.

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PAPER II

The fatty acid binding protein 7 (FABP7) is involved in proliferation and invasion of melanoma cells

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The fatty acid binding protein 7 (FABP7) is involved in proliferation and invasion of melanoma cells

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Abstract

Background: The molecular mechanisms underlying melanoma tumor development and progression are still not completely understood. One of the new candidates that emerged from a recent gene expression profiling study is *fatty acid-binding protein 7* (*FABP7*), involved in lipid metabolism, gene regulation, cell growth and differentiation.

Methods: We studied the functional role of FABP7 in human melanoma cell lines and using immunohistochemistry analyzed its expression pattern and clinical role in 11 nevi, 149 primary melanomas and 68 metastases.

Results: FABP7 mRNA and protein level is down-regulated following treatment of melanoma cell lines with a PKC activator (PMA) or MEK1 inhibitor (PD98059). Down-regulation of FABP7 using siRNA decreased cell proliferation and invasion but did not affect apoptosis. In clinical specimens, FABP7 was expressed in 91% of nevi, 71% of primary melanomas and 70% of metastases, with a cytoplasmic and/or nuclear localization. FABP7 expression was associated with tumor thickness in superficial spreading melanoma (P = 0.021). In addition, we observed a trend for an association between FABP7 expression and Ki-67 score (P = 0.070) and shorter relapse-free survival (P = 0.069) in this group of patients.

Conclusion: Our data suggest that FABP7 can be regulated by PKC and the MAPK/ERK1/2 pathway through independent mechanisms in melanoma cell lines. Furthermore, FABP7 is involved in cell proliferation and invasion *in vitro*, and may be associated with tumor progression in melanoma.

Background

Malignant melanoma is the most lethal skin cancer and accounts for about 75% of all deaths from skin tumors. In its early stage, melanomas can be treated surgically, but once the tumor has progressed, it is difficult to treat and it does not respond to current therapies. The molecular

mechanisms underlying melanoma development and progression are still not completely understood, and novel diagnostic and prognostic markers, as well as therapeutic targets are needed [1].

We have previously reported that phorbol-12-myristate-13-acetate (PMA), a protein kinase C (PKC) activator, increases proliferation and promotes anchorage-independent survival of melanoma cells cultivated as multicellular aggregates in suspension (spheroids). This protective action was at least partly mediated through PKC and MEK-independent activation of the mitogen-activated protein kinase/extracellular signal-regulated kinases 1/2 (MAPK/ERK1/2) [2]. In an attempt to identify additional genes involved in survival and apoptosis of melanoma cells, we used high throughput gene expression profiling (Affymetrix™) to identify differentially expressed genes in untreated cells cultured as monolayer or spheroids, as well as in spheroids treated with PMA and/or the MEK1 inhibitor PD98059. The analysis revealed that the *fatty acid-binding protein 7 (FABP7, BLBP* or *B-FABP)* [3,4] was among the most significantly differentially expressed genes (unpublished results).

FABP7 belongs to a family of structurally-related proteins showing tissue-specific patterns of expression. Nine FABPs (FABP1 – FABP9), expressed in normal liver, intestine, heart, adipose tissue, epidermis, brain, peripheral nervous system and testis have been identified (reviewed in [5]). FABP proteins are involved in lipid metabolism, including uptake and intracellular trafficking of fatty acids and retinoids. In addition, they play a role in gene regulation, cell signaling, cell growth and differentiation [6].

Several reports have suggested a possible role for the different FABP proteins in cancer biology, linking their levels with either increasing or decreasing degree of malignancy. Adamson et al. reported that FABP5 (C-FABP/E-FABP) protein expression is higher in prostate cancer compared to prostatic hyperplasia [7]. On the other hand, the FABP1 level decreases with progression of colon cancer [8]. FABP7 is highly expressed in glia cells throughout development of the nervous system [4,9] and high FABP7 expression in glioblastomas is related to poor prognosis [10]. Recently, two studies have addressed FABP7 expression in surgical specimens from melanoma patients. While de Wit et al. reported down-regulation of FABP7 in melanomas compared to nevi, Goto et al. found FABP7 to be frequently expressed in melanomas, and suggested that it may play a role in cell proliferation and invasion[11,12].

In the current study we examined the role of FABP7 in proliferation, apoptosis and invasion of melanoma cells grown *in vitro* and studied possible regulation mechanisms of this protein. In addition, we examined the expression of FABP7 protein in clinical melanoma specimens and assessed the relationship between FABP7 expression pattern and known prognostic variables, cell cycle factors and disease progression. We report that FABP7 is regulated via PKC and the MAPK/ERK1/2 signaling pathway in melanoma cells *in vitro* and promotes proliferation and invasion. Moreover, FABP7 expression is associated with tumor thickness and proliferation in melanoma biopsies.

Methods

Cell lines and Growth Conditions

The Wistar Melanoma (WM) cell lines were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and have been described in detail elsewhere [13].

The MeWo cell line was derived from a lymph node metastasis [14]. The cell lines FEMX-I and LOX were established from metastatic lymph node biopsies obtained from melanoma patients treated at the Rikshospitalet-Radiumhospitalet Medical Center [15]. The cells were routinely cultured in RPMI 1640 medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) (Biochrom, KG, Berlin, Germany). Phorbol-12-myristate-13-acetate (PMA) was from Sigma-Aldrich (St. Louis, MO), whereas the MEK1 inhibitor, PD98059, was from Cell Signaling Technology (Beverly, MA). Multi-cellular aggregates (spheroids) were prepared as previously described [16]. Briefly, 24-well plates were coated with 1% Seaplaque agarose (BioWhittaker Molecular Application, Rockland, ME) and tumor cells (2×10^5 cells in 1 ml complete medium) were plated on top of the solidified agarose. For thymidine incorporation assay, 5000 cells per well were plated in 96-well polyhema (Sigma-Aldrich)-coated U-bottom plates. For treatment of spheroid cultures, PMA was added when plating in suspension, whereas the inhibitors in combination experiments were added 45 min prior to plating as spheroids.

Gene expression analysis

WM35 cells were grown as spheroids for 24 hrs in the presence of PMA and PD98059, alone and in combination. Total RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA). Gene expression profiling was performed using Affymetrix U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). For microarray hybridization, the protocol described in the Affymetrix GeneChip eukaryotic one-cycle target preparation protocol, using 5 μg of total RNA, was followed. Analysis of the data was performed by Genolyze Ltd. (Turku, Finland) using statistical software R version 2.3.0. and package collection Bioconductor version 1.8. Statistical significance was assessed using p-value

from two-tailed two sample t-test. P-values are replaced with q-values to control the False Discovery Rate.

Quantitative real time RT-PCR analysis

The high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA) was used to reverse-transcribe total RNA (0.8 μg) in a 20 μl reaction mixture using random primers. The real-time PCR analyses were performed using TaqMan Fast Universal PCR Master Mix (2×) and TaqMan Gene Expression Assay (HS00361426-ml FABP7, HS99999908-ml GUS, Applied Biosystems). A total of 0.5 μl cDNA was used in 25 μl PCR mixtures with 900 nM of each primer and 250 nM TaqMan probe. The reactions were carried out in a 7900 HT Fast Real Time PCR system (Applied Biosystems) with the following program: 95°C for 20 sec. followed by 40 cycles of 95°C for 1 sec., 60°C for 20 sec. Each sample was run in triplicate. The *FABP7* relative mRNA expression level was normalized with respect to the beta-glucuronidase (GUS) gene, which had stable transcript levels under these experimental conditions. The mean from three independent experiments was calculated.

Immunoblotting

Cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40, 10% glycerol, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 100 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin, and 10 μl/ml phosphatase inhibitor cocktail I and II (Sigma-Aldrich)). Protein quantitation was done by Bradford analysis and 25 μg protein/lane was resolved by SDS polyacrylamide gel electrophoresis. Transfer and hybridization were as described in [17]. To ensure even loading, filters were stained with naphthol-blue black (Sigma-Aldrich) and re-stained with α-tubulin. The antibodies against FABP7 and α-tubulin were from R&D Systems (Minneapolis, MN) and Calbiochem (San Diego, CA), respectively. HRP-conjugated anti-mouse IgG secondary antibody was from Promega (Madison, WI) and HRP-conjugated anti-goat secondary antibody was from DAKO A/S (Glostrup, Denmark).

Small interfering RNA transfection

Fifty thousand cells per well were seeded in 24-well plates for 24 hrs prior to transfection with 50 nM siRNA targeting FABP7 (OligioID: HSS103516; Catalog# 1299003) or negative control siRNA duplexes (Catalog#12935-300) using Lipofectamine™ RNAiMAX transfection reagent (all reagents and siRNA were from Invitrogen). Cells were detached 48 hours after transfection and plated into agarose-coated 24-well plates as spheroids for an additional 72 hrs for assessment of apoptosis, seeded into 96-well polyhema-coated U-bottom plates for the proliferation assay and plated in BioCoat Matrigel invasion chambers.

Proliferation assay

Five thousand cells per well were seeded in 96-well polyhema-coated U-bottom plates for spheroids and in 96-well flat-bottom plates for monolayer cells and cultured for 72 hrs, the last 24 hrs with the addition of 3.7×10^4 Bq [^{3}H]Thymidine (ARC, St.Louis, MO) Thereafter, the cells were harvested using a Filtermate Harvester (Packard Instrument Co. Meriden, CT). [^{3}H]Thymidine incorporation was assessed in a Packard Microplate Scintillation Counter. Proliferation assays were measured in triplicate. The experiment was repeated at least three times.

Flow cytometric analysis of apoptosis

The adherent cells were harvested by Trypsin and together with detached cells fixated in 100% cold methanol. Fixed cells were washed with PBS, incubated for 30 min at 37°C in 50 μl terminal transferase (TdT) solution containing 5 units TdT (Roche, Basel, Switzerland), 10 μl 5× reaction buffer (supplied with TdT), 1.5 mM $CoCl_2$, 0.5 nmol labeled biotin-16-dUTP, 0.1 mM dithiothreitol and distilled water. The cells were subsequently washed once in PBS containing 0.1% Triton X-100 and incubated in 50 μl 1:50 streptavidin-FITC (Amersham, Buckinghamshire, UK) in PBS (0.1% Triton X-100) and 3% skimmed dry milk for 45 min at room temperature. After washing in PBS (0.1% Triton X-100) the pellet was resuspended in PBS (0.1% Triton X-100) containing 2 μg/ml Hoechst 33258 to a final concentration of 1×10^6 cells/ml and incubated for 30 min at 4°C. Data acquisition and analysis were performed on Becton Dickinson LARII (Becton Dickinson immunocytometry systems, San Jose, CA) using Multifit software (FACSDiVa House inc., Tonsham, ME).

Matrigel invasion assay

WM35 and WM239 cells were plated in BioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA) at a cell density of 3×10^4 per chamber in RPMI 1640 supplemented with 5% fetal bovine serum (inner chamber) 48 hrs post-transfection. Self-supplied fibroblast conditioned medium was used as chemoattractant in the outer chamber. The conditioned medium was obtained from fibroblasts isolated as described by Costea *et al*[18] cultivated in DMEM supplemented with 10% fetal bovine serum. The medium was collected when the cells were 70% confluent. After 48 hrs incubation at 37°C and 5% CO_2, non-invading cells remaining on the top surface of the chamber were removed by scrubbing with a cotton-tipped swab, and the invading cells that had adhered to the bottom surface of the chamber membranes were fixed, stained with hematoxylin and counted.

Clinical melanoma specimens

Formalin-fixed, paraffin-embedded tissue from 149 primary and 68 metastatic melanomas, as well as 11 benign

nevi, was examined for expression of FABP7 protein. Of the primary tumors, 93 were classified as superficial spreading (SSM) and 56 as nodular melanomas (NM). Clinical follow-up was available for all patients. The study was approved by the Regional Committee for Medical Research Ethics in Norway.

Immunohistochemical analysis

Sections of formalin-fixed, paraffin-embedded tissue were immunostained using the two-step EnVision system (DAKO EnVision™, DAKO A/S). Deparafinized sections were microwaved in low pH buffer (pH 6.0) (DAKO) at 750 W for 5 minutes and then at 500 W for 15 minutes to unmask the epitopes. After treatment with 1% hydrogen peroxide for 5 minutes to block endogenous peroxidase, the sections were incubated with polyclonal rabbit anti-human FABP7 antibody (R&D Systems) for 30 minutes at room temperature followed by 30 minutes incubation with mouse anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were then incubated with HRP-labeled secondary antibody for 30 minutes followed by 5 minutes incubation at RT with DAB substrate (DAKO A/S). All series included positive controls. Four semiquantitative classes were used to describe the number of stained cells: negative, ≤ 5%, 6–50% and >50%. Both nuclear and cytoplasmic staining was scored. Staining was evaluated by a surgical pathologist (BD). A subset of the cases (n = 50) was additionally scored by another author (AS).

Statistical analysis

Statistical analysis was performed using the SPSS program version 13.0 (Chicago, IL). The differences between FABP7 expression in benign nevi, primary melanomas and metastases were analyzed using the Chi-square test. The relationship between FABP7 expression and mean tumor thickness was evaluated nonparametrically using the Mann-Whitney two sample test. The association between expression of FABP7 and cell cycle markers was performed using the Fischer's exact test. Kaplan-Meyer estimates and the log-rank test were used for survival analysis. $P < 0.05$ was considered statistically significant.

Results

Identification of molecules involved in survival of melanoma cells as multicellular aggregates in suspension using gene expression profiling

We previously showed that PMA treatment protects melanoma cells from suspension-mediated apoptosis while the MEK1 inhibitor PD98059 has the opposite effect [2]. In order to identify new factors involved in anchorage-independent growth of melanoma cells, we compared mRNA expression profiles from the melanoma cell line WM35, cultured in monolayer or as untreated spheroids, as well as following treatment of the spheroids with PMA and/or PD98059 for 24 hours.

The *FABP7* gene was among the genes showing the highest differential expression. While no notable difference was observed between monolayer cells and spheroids, treatment with PMA or PD98059, as well as with PD98059 and PMA in combination, led to *FABP7* mRNA down-regulation in treated spheroids compared to the spheroid control (Figure 1a). The microarray results were validated using real time RT-PCR (Figure 1b).

FABP7 is expressed in melanoma cell lines and regulated through PKC and the MAPK/ERK1/2 signaling pathway

The protein level of FABP7 in monolayer culture, untreated spheroids and spheroids treated with PMA and/or PD98059 for 24 hrs was analyzed using western blot. As shown in Figure 1c, no change in FABP7 protein level was observed between monolayer cells and untreated spheroids while in spheroids treated with PMA and/or PD98059, the protein level was reduced compared to controls. This was in accordance with the reduction of FABP7 mRNA levels. A similar reduction in FABP7 protein level was obtained in monolayer cultures treated with PMA and/or PD98059 (data not shown).

In order to reveal if FABP7 expression levels differ during the cultivation of the WM35 cells following PMA and/or PD98059 treatment, we performed a time course study. The monolayer cells were treated with PMA or PD98059 from 0,5 hrs to 72 hrs. As shown in Figure 2. we observed down-regulation of FABP7 protein after 12 hrs in both PMA and PD98059 treated cells although the effect of PMA was more pronounced over time. The down-regulation was sustained for up to 72 hrs for both treatments. These results were supported by real time RT-PCR (data not shown).

To examine if FABP7 is frequently expressed in melanoma cell lines we analyzed the level of FABP7 mRNA and protein in two primary (WM1341 and WM902B) and seven metastatic cell lines (WM239, WM45.1, WM983, WM9, LOX, MeWo and FEMX-I) in addition to WM35. As shown in Figure 3a and 3b, variable levels of FABP7 mRNA and protein were detected in 9 out of 10 cell lines. With the exception of WM45.1, good concordance between mRNA and protein levels was observed in all the cell lines. No clear differences were observed between FABP7 expression levels in cell lines originating from primary tumor vs. metastasis.

FABP7 is involved in proliferation and invasion of melanoma cells

In order to further investigate the function of FABP7 we chose to transiently down-regulate FABP7 using specific

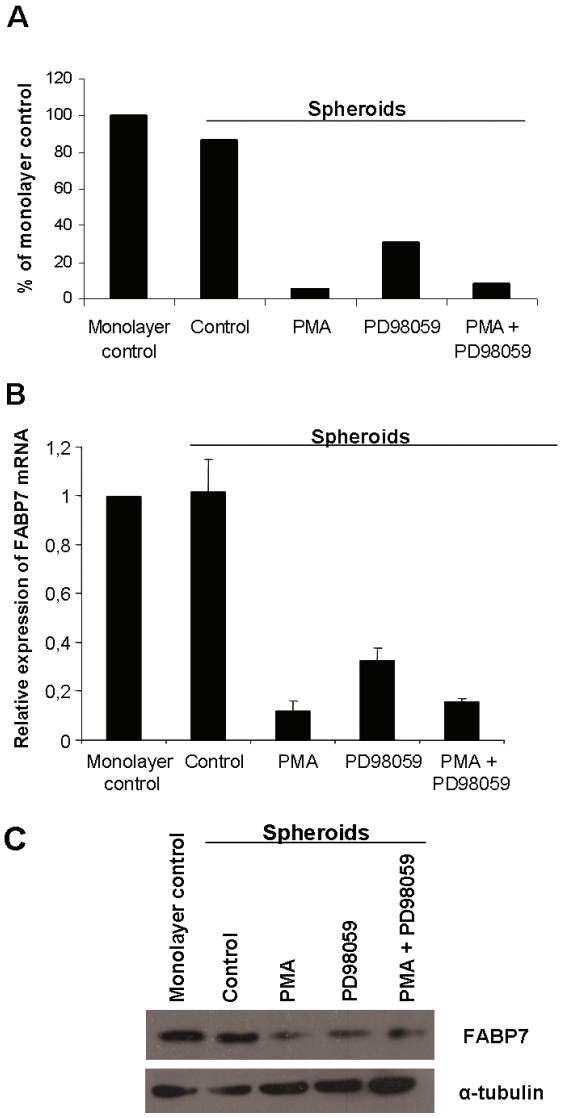

Figure 1

A) The expression level of FABP7 as detected by Affymetrix microarray analysis in WM35 cells grown as spheroids for 24 hrs with or without PMA and/or PD98059 compared to untreated monolayer control. B) *FABP7* mRNA expression levels in the same cells as in (A) measured by real time RT-PCR. The results presented are relative to untreated monolayer control. The average was calculated from three independent experiments and presented with standard deviation. C) Expression of the FABP7 protein by Western blot analysis. Down-regulation of *FABP7* mRNA and protein was seen after treatment with PMA and PD98059 for 24 hrs. α-tubulin was used as loading control.

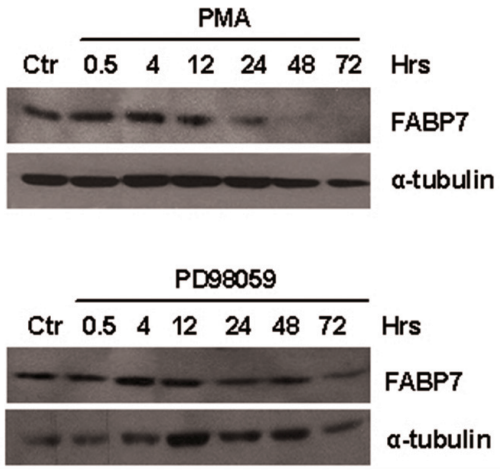

Figure 2
Western blot showing the expression of the FABP7 protein in WM35 monolayer cells treated with PMA or PD98059 for 0.5 hrs, 4 hrs, 12 hrs, 24 hrs, 48 hrs and 72 hrs. α-tubulin was used as loading control.

siRNA in the WM35 and WM239 cell lines, which we found to have high FABP7 expression. The effect of down-regulation on proliferation, invasion and apoptosis was examined. Monolayer cells were incubated for 48 hrs with FABP7 siRNA or a control siRNA and analyzed for transfection efficiency by western blot (Figure 4a). As demonstrated in figure 4b and 4c, FABP7 down-regulation reduced proliferation by 29% in WM35 and 84% in WM239 cells as compared to scrambled siRNA control transfected cells. Similar results were obtained when the cells were grown in suspension (data not shown).

The degree of apoptosis was assessed using TdT-mediated dUTP nick end labeling (TUNEL) staining and flow cytometry. Analysis of both monolayer and spheroid cultures showed that down-regulation of FABP7 did not affect the percentage of apoptotic cells (data not shown). Together these results suggest that FABP7 is most likely involved in proliferation and not apoptosis in melanoma cells.

We investigated the effect of *FABP7* down-regulation on invasion using the Matrigel assay. The number of invading cells was reduced by 55% and 40% in WM35 and WM239 cell respectively after transfection with FABP7 siRNA compared with scrambled siRNA control-transfected cells (Figure 4d), suggesting that FABP7 contributes to the invasiveness of melanoma cells.

FABP7 is expressed in melanomas and associated with tumor thickness

In order to examine the clinical relevance of FABP7, paraffin-embedded tissue from a panel of benign nevi and primary and metastatic melanomas was analyzed for expression of FABP7 protein using immunohistochemistry. Heterogeneous cytoplasmic and/or nuclear expression of FABP7 was observed in 91% of the nevi, 71% of the primary tumors and 70% of the metastases. The results are summarized in Table 1 and 2 and illustrated in Figure 5. Statistical analysis demonstrated a significant higher cytoplasmic FABP7 expression in nevi compared to primary and metastatic melanomas (P = 0.023), with comparable nuclear expression. A two-tier analysis of primary and metastatic melanomas showed comparable expression for both cytoplasmic and nuclear expression (P > 0.05). Good concordance (>80%) was achieved between the two observers. Discrepant cases were resolved through a consensus session.

Since 62% (92/149) of the primary tumors expressed cytoplasmic FABP7 in more than 5% of the cells, this cut-off was used to distinguish between high and low protein levels. Applying the same cutoff when evaluating nuclear staining, we observed that only 13% (19/149) of the tumors had high protein expression levels. Higher cytoplasmic FABP7 was significantly associated with increased thickness of SSM (P = 0.021). In addition, in this group of patients, a trend towards increased relapse-free survival (P = 0.069) for patients whose tumors expressed less FABP7 was observed. No such correlation was observed in NM (Table 3 and Figure 6). We did not observe any significant correlation between FABP7 staining and overall survival for patients diagnosed with either SSM or NM (data not shown). Nuclear staining had no association with disease outcome (data not shown).

Relationship between FABP7 expression and markers of proliferation

Since our panel of primary and metastatic melanomas has previously been analyzed for expression of cell cycle progression markers [19-21] and activation status of MAPK/ERK1/2 [22], it was of interest to examine the relationship between FABP7 expression and the levels of these factors. The results showed no correlation between cytoplasmic FABP7 expression and the expression of the cyclins A, D1 or D3 or the cdk inhibitors $p21^{CIP1/WAF1}$and $p27^{Kip1}$ in either SSM or NM. However, a trend for an association between cytoplasmic FABP7 and Ki-67 (P= 0.07) in SSM was observed, which is in support of our *in vitro* results, suggesting FABP7 involvement in proliferation. Furthermore, expression of activated MAPK/ERK1/2 did not cor-

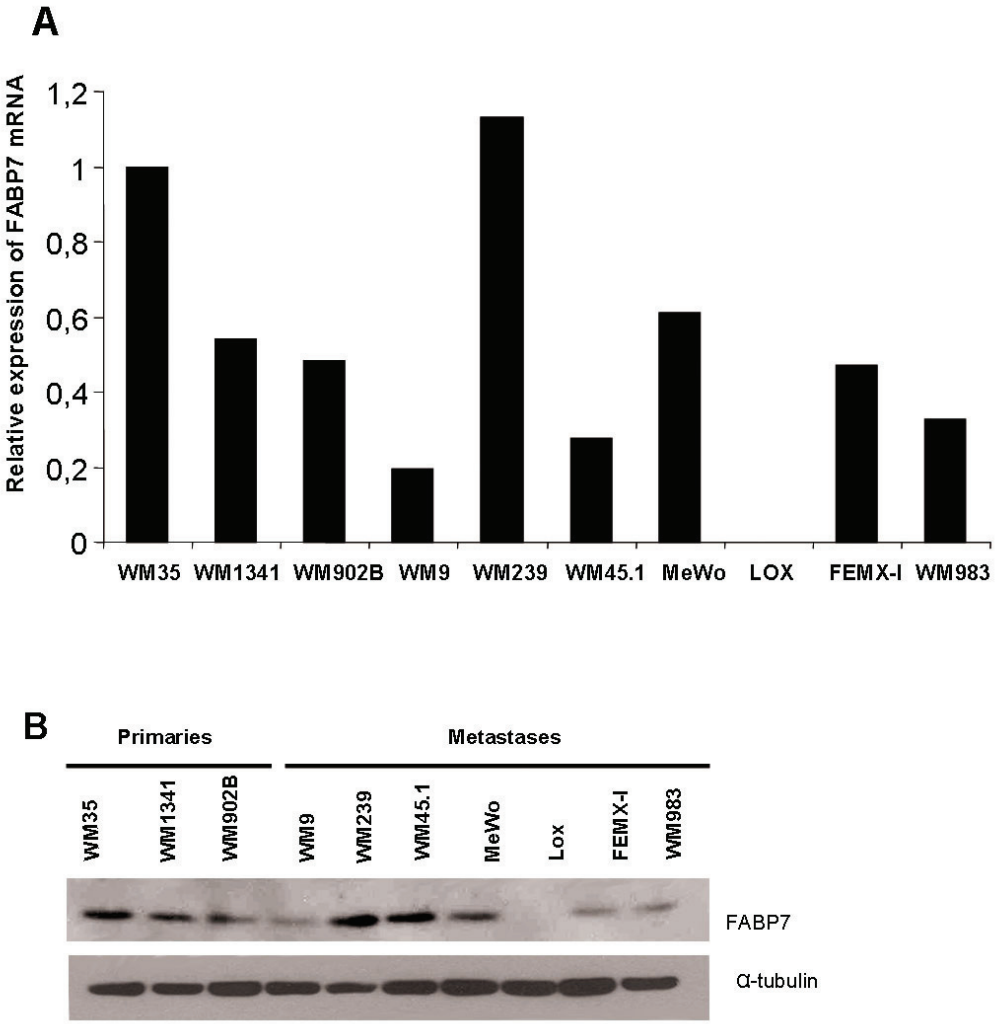

Figure 3
A) *FABP7* mRNA level in melanoma cell lines as measured by real time RT-PCR analysis. Three primary and 7 metastatic melanoma cell lines were evaluated. The expression levels are shown relative to the WM35 cell line. B) Expression of FABP7 protein by western blotting, with α-tubulin as loading control.

relate with FABP7 expression. Interestingly, however, we observed that if the cutoff level was changed (only total lack of FABP7 was regarded as low expression), MAPK/ERK1/2 expression did positively correlate with cytoplasmic FABP7 expression in SSM (P= 0.047). This was not the case for NM, regardless of cutoff. Nuclear expression of FABP7 did not correlate with any of the examined markers (data not shown).

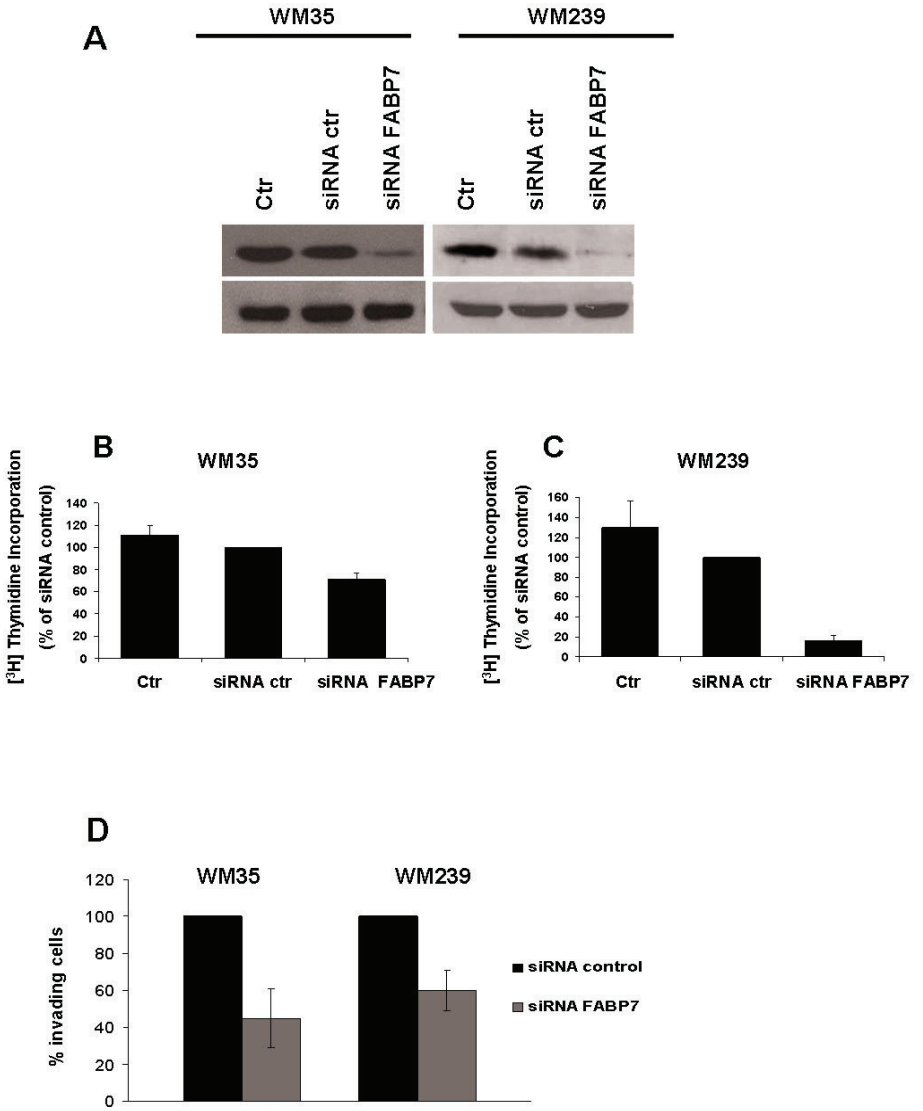

Figure 4

A) Western blot analysis showing down-regulation of FABP7 in WM35 and WM239 cells after transfection with FABP7 siRNA, with α-tubulin as loading control.B-C) The effect of FABP7 down-regulation on proliferation in WM35 (B)and WM239 (C)cells measured by [^{3}H]Thymidine incorporation 72 hrs post transfection. Down-regulation of FABP7 protein using siRNA led to reduction of DNA synthesis, suggesting reduced proliferation in both cell lines.D) Matrigel invasion assay. Inhibition of invasion ability of WM35 and WM239 cells following FABP7 down-regulation with siRNA. The average was calculated from three independent experiments and presented with standard deviation.

Table 1: Number (percentage) of melanocytic lesions expressing FABP7 in different cellular compartments

Tumor Type	No. of tumors	Total no. of positive	Cytoplasm	Nucleus	Cytoplasm/nucleus
Benign nevi	11	10 (91)	2 (18)	0 (0)	8 (73)
Primary melanomas	149	106 (71)	36 (24)	0 (0)	70 (47)
SSM	93	61 (66)	19 (21)	0 (0)	42 (45)
NM	56	45 (80)	17 (30)	0 (0)	28 (50)
Metastases	68	48 (70)	20 (29)	1 (2)	27 (39)

Discussion

We previously showed that PMA-mediated PKC activation and activation of the MAPK/ERK1/2 pathway contributes to increased proliferation and reduced apoptosis of melanoma cells under anchorage-deprived conditions. In the present study we used gene expression profiling to identify additional genes involved in these processes, and found the *FABP7* gene to be differentially expressed and down-regulated in WM35 spheroids following both PKC activation and MEK1 inhibition while no differences were observed between monolayer cells and untreated spheroids. PKC activation and MAPK/ERK1/2 down-regulation had opposite effect on anchorage-independent survival of the melanoma cells, but both negatively regulated FABP7. This observation argues against FABP7 involvement in promotion of anchorage-independent survival in these cells. Thus, it is likely that these pathways are regulating additional factors important for survival, independent of FABP7 down-regulation.

PKC is a well known activator of the MAPK/ERK1/2 pathway [23,24] and we have previously reported that PMA activates MAPK/ERK1/2 independently of its upstream activator MEK1 [2]. Since PMA treatment down-regulates FABP7 even in the presence of activated ERK1/2 this down-regulation is likely to be PMA/PKC-mediated but MAPK/ERK1/2-independent. Together this suggests that FABP7 can be regulated by both signaling pathways independently in melanoma cells. Several reports have shown that activation of the MAPK/ERK1/2 pathway can induce increased activity of peroxisome proliferator-activated receptors α/γ (PPAR α/γ). Similarly, PKC can both positively and negatively regulate PPARα-dependent transcription [25-27]. Binding of PPARγ to its response element, PPRE, has been shown to up-regulate FABP1 and FABP4 [5,28,29]. It is reasonable, therefore, to assume that FABP7 might also be regulated through this mechanism.

To further clarify the role of FABP7 in melanomas we used siRNA to down-regulate its expression in the primary WM35 and metastatic WM239 melanoma cell lines. This down-regulation notably inhibited proliferation in both cell lines, but did not affect the degree of apoptosis, arguing for involvement of FABP7 in melanoma proliferation. In support of our results, Goto *et al* [12] showed that proliferation of melanoma cell lines is reduced upon down-regulation of FABP7, also without affecting apoptosis. Our results showed that down-regulation of FABP7 negatively influences the invasive potential of melanoma cells, also in agreement with Goto *et al* [12] who demonstrated that down-regulation of FABP7 decreased invasiveness in 2 of 6 melanoma cell lines. In further support of this hypothesis are the data of Mita *et al*., who showed that FABP7 increases the invasion properties of astrocytoma cells [30]. Of note, when FABP7 was reintroduced in the metastatic cell line LOX, lacking constitutive FABP7 expression, no effect on apoptosis, proliferation or invasion was observed (preliminary results, data not shown). Similar results were reported by Goto *et al*. [12] in 4 out of 6 melanoma cell lines. However, the reason for the discrepancy between the cell lines is still unclear. Thus, the biological role and detailed functional mechanism of the FABP7 protein in melanoma cells remains to be further investigated.

Table 2: Number (percentage) of melanocytic lesions expressing different levels of FABP7

Tumor type	No. of tumors	Cytoplasm				Nucleus			
		-	≤ 5%	6–50%	> 50%	-	≤ 5%	6–50%	> 50%
Benign nevi	11	1 (9)	0 (-)	1 (9)	9 (82)	3 (28)	4 (36)	4 (36)	0 (-)
Primary melanomas	149	43 (28)	14 (9)	42 (28)	50 (34)	79 (53)	51 (34)	16 (11)	3 (2)
SSM	93	32 (34)	10 (11)	25 (27)	26 (28)	51 (55)	31 (33)	9 (10)	2 (2)
NM	56	11 (20)	4 (7)	17 (30)	24 (43)	28 (50)	20 (36)	7 (12)	1 (2)
Metastases	68	21 (31)	8 (11)	21 (31)	18 (27)	40 (59)	20 (29)	7 (11)	1 (1)

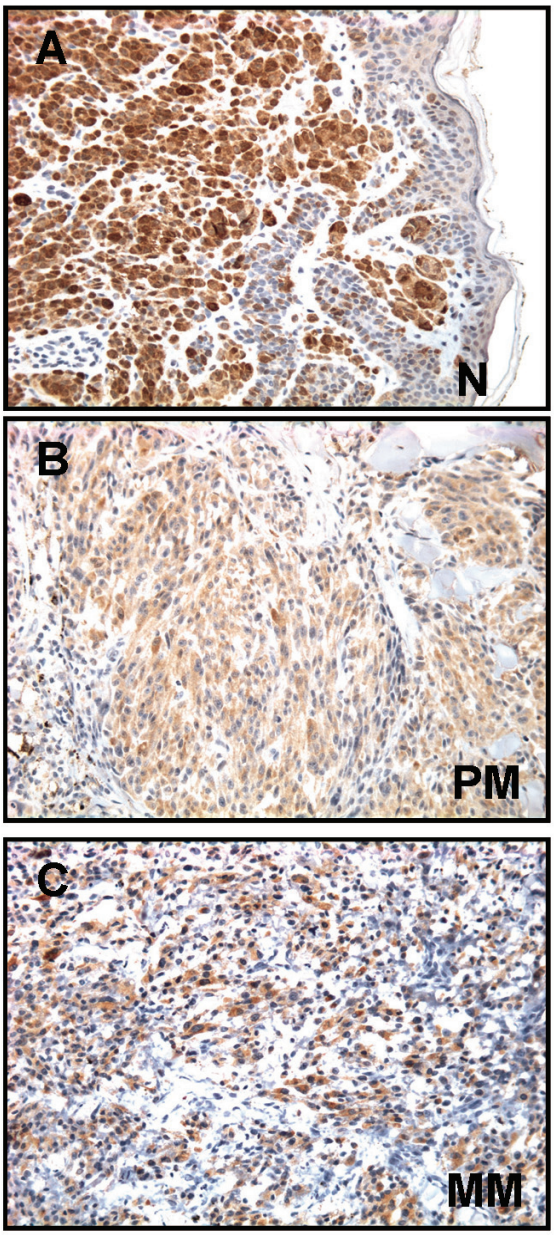

Figure 5
Immunohistochemical staining of FABP7 in a benign nevus (A) primary melanoma (B) and metastatic melanoma (C).

Table 3: Relationship between cytoplasmic FABP7 expression and tumor thickness in primary melanomas

Tumor type	Expression	No of patients	Average depth of growth (mm)	*P*
SSM	Negative*	41	1.59	
	Positive	48	2.17	0.021
NM	Negative*	15	4.32	
	Positive	41	4.78	0.697

* Considered negative if < 5% tumor cells showed positive immunoreactivety of FABP7

Several members of the FABP family have been reported to be differentially expressed in cancer. Loss of expression of FABP4 was reported in bladder cancer while FABP1 and FABP2 are over-expressed in prostate and breast cancers [7,31-33]. In accordance with Goto *et al.* [12] we found that FABP7 is expressed in both primary and metastatic melanoma cell lines, as well as in melanocytic lesions. However, there were no clear differences in FABP7 expression levels in primary derived compared to metastatic derived cell lines, suggesting that FABP7 is not associated with tumor aggressiveness. On the other side, cell lines are cultured in artificial environments that can not be directly compared to tumors *in vivo* and a connection to tumor aggressiveness and progression can not be completely excluded. In support of this, analysis of the clinical data showed that thicker SSM expressed higher levels of FABP7. Furthermore, a trend between high levels of FABP7 and reduced disease-free survival for these patients suggest that FABP7 could contribute to disease progression, possibly by increasing the invasion potential of the tumors. In support of our results, a negative association between FABP7 expression and survival was recently observed for patients with glioblastoma [10,34]. We also observed a positive trend between FABP7 and the proliferation

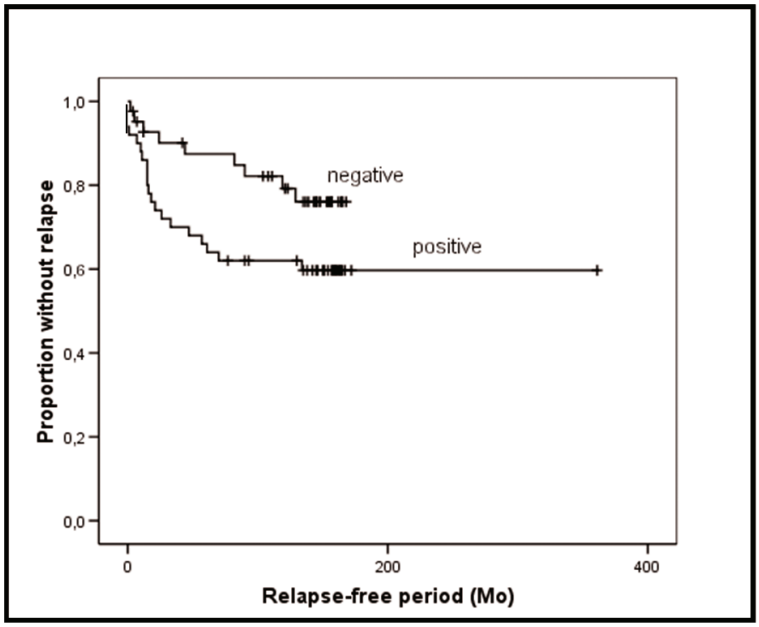

Figure 6
Kaplan-Meier curve demonstrating a negative trend (*P* = 0.069) between protein expression of cytoplasmic FABP7 and relapse-free survival for patients with SSM. FABP7 expression was considered high when > 5% of the tumor cells showed positive staining with the anti-FABP7 antibody.

marker Ki-67 in SSM, suggesting that FABP7 may contribute to increased proliferation *in vivo*. Since the patient subgroups in the analyses were small, the suggested clinical significance of FABP7 expression remains to be confirmed in larger patient cohorts.

In the clinical specimens, FABP7 protein expression was highest in nevi, with no observed differences between primary and metastatic melanoma. This is in accordance with the study by de Wit *et al*. [11] who reported that FABP7 is down-regulated in melanoma tissue compared to nevi using oligonucleotide arrays. The higher expression of FABP7 in nevi compared to melanomas seems contradictory to the *in vitro* data in the present study, as well as to the association with clinical parameters of disease progression. We are unable to explain this discrepancy at present. However, the majority of benign nevi are terminal lesions that do not progress to melanoma and the molecular events regulating these processes might differ. It is also possible that different expression levels of FABP7 mediate different effects during disease progression.

Variation in sub-cellular localization of FABP7 has been reported in developing radial glia cells, glioma cell lines [9,35] and glioblastoma multiforme (GBM) specimens [34]. Since FABP proteins are considered to be co-activators in PPAR-mediated gene transcription control, this could in part explain FABP7 translocation to the nucleus (reviewed in [5]). Recently, it was reported that nuclear expression of FABP7 is restricted to infiltrative tumor types and related to EGFR amplification and over-expression as well as poor prognosis of GBM [10,34]. In our melanoma cohort we did not find any association between nuclear expression of FABP7 and disease-free or overall survival.

Conclusion

We confirmed that FABP7 protein is expressed in melanocytic lesions and showed that it can regulate proliferation and invasion in melanoma cells *in vitro*. Our results further suggest that FABP7 can be regulated by PKC and the MAPK/ERK1/2 pathway through independent mechanisms. In addition, FABP7 expression is associated with proliferation and tumor thickness in the patients with SSM, suggesting that for these patients FABP7 could be a potential target for therapy.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS carried out the siRNA transfections, invasion experiments and evaluated immunohistochemical staining and drafted the manuscript. KJ performed immunohistochemistry, flow cytometry, and contributed to drafting the manuscript. MS and AKRR carried out optimalization of the real-time PCR and antibodies used in western blotting in the study. MS also performed technical phase of microarray experiment. BD evaluated immunohistochemical staining. GT participated in the design of the study and microarray analysis. VAF conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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PAPER III

Biological effects induced by insulin-like growth factor binding protein 3 (IGFBP-3) in malignant melanoma.

Oy GF, Slipicevic A, Davidson B, Solberg Faye R, M Mælandsmo G, Flørenes VA.

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APPENDIX

ABBREVATIONS

Akt	V-Akt murine thymoma viral oncogene homolog
AP1	Activator protein 1
APAF-1	Apoptosis protease activationg factor-1
ARF	Alternative reading frame of the INK4 locus
ATF1/2	Activating transcription factor 1 or 2
Bad	Bcl-2-associated death promoter homolog
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell chromic lymphocytic leukemia/lymphoma-2 protein
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 like 11 protein
Bcl-$_X$S/$_X$L	Bcl-2 like1 protein (short form/long form)
B-RAF	V-raf murine sarcoma viral oncogene homolog B1
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CDKN2A	Cyclin dependent kinase inhibitor 2A
c-fos	Cellular fos proto-oncogene
c-jun	Cellular jun proto-oncogene
c-myc	Cellular myc proto-oncogene
CREB	cAMP responsive element binding protein
DISC	Death-inducing signal complex
DNA	Deoxyribonucleic acid
E2F	E2F transcription factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGR1	Early growth response 1
EGFR	Epidermal growth factor receptor
Elk1	ELK1, member of ETS oncogene family
ERK	Extracellular signal-regulated kinases
Ets1	v-ets erythroblastosis virus E26 oncogene homolog 1

FABP	Fatty acid binding protein
FAK	Focal adhesion kinase
FADD	Fas-associated death domain protein
Fas	TNF receptor superfamily, member 6
FasL	Fas lignad
FGFR	Fibroblast growth factor receptor
GSK3-β	Glycogen synthase kinase-3 beta
IAP	Inhibitor of apoptosis
ICAT	Beta-catenin-interacting protein
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP	Insulin-like growth factor binding protein
IL-1	Interleukin-1
ILK	Integrin-linked kinase
INK4	Inhibitor of cyclin dependent kinase 4
IRS	Insulin receptor substrate
JNK	c-jun N-terminal kinase
kDa	kilo Dalton
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LEF1	Lymphoid enhancer binding factor-1
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Mouse double minute 2
MEF2A	Myocyte enhancer factor 2A
MITF	Microphtalmia-associated transcriptional factor
MMP	Matrix metalloproteinase
MK2	MAPK-activated kinase 2
MNK1/2	MAPK interacting serine/threonine kinase 1 and 2
MSK1	Mitogen-and stress-activated protein kinase 1
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NFAT4	Nuclear factor of activated T-cells 4
NFκ-B	Nuclear factor kappa-B
NGF	Nerve growth factor

NR4a1	Nuclear receptor subfamily 4, group A, member 1
H-RAS	Human homolog to Harvey rat viral sarcoma oncogene, encoding RAS
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
PDGFR	Platelet-derived growth factor receptor
PDK1 and 2	Phosphoinositide–(3,4,5)-triphosphate-dependent-kinases 1and 2
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2
PMA	Phorbol-12-myristate-13-acetate
PP2	4-Amino-5-(4-chlorphenyl)-7-(t-butyl)(3,4-d) pyrimidine
PPAR	Peroxisome proliferators-activated receptor
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
RACK1	Receptor for activated kinase C 1
RB	Retionoblastoma
RTK	Receptor tyrosine kinase
RGF	Radial growth phase
RXR	Retinoid X receptor
SA-β-GAL	Senescence associated acidic β-galactosidase
Sap-1a	Serum response factor (SRF) associated protein 1
Shc	Src homology 2 domain containing
siRNA	Small interfering RNA
Smac/Diablo	Second mitochondria-derived activator of caspases/Direct IAP-binding protein
SMAD4	Mothers against decapentaplegic homolog 4
Src	Human homolog to the avian v-Src gene of the Rous Sarcoma virus
STAT1/3	Signal transducer and activator of transcription 1 and 3
Tau	Microtubule-associated protein Tau
TGF- α /ß	Transforming growth factor-alpha/beta
TNF-α	Tumor necrosis factor-alpha
TPA	12-O-tetradecanoyl phorbol 13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase end labeling
UV	Ultra violet

VEGFR	Vascular endothelian growth factor receptor
VGF	Vertical growth phase
Wnt	Wingless-type MMTV integration site family